

12

EUROPEAN PATENT APPLICATION

21 Application number: 85102250.9

22 Date of filing: 28.02.85

51 Int. Cl.⁴: **C 07 K 7/04, A 61 K 39/29,**
A 61 K 37/02, C 07 K 17/02,
G 01 N 33/577, C 07 K 1/06,
A 61 K 39/385

30 Priority: 07.03.84 US 587090
05.02.85 US 698499

43 Date of publication of application: 18.09.85
Bulletin 85/38

84 Designated Contracting States: AT BE CH DE FR GB IT
LI NL SE

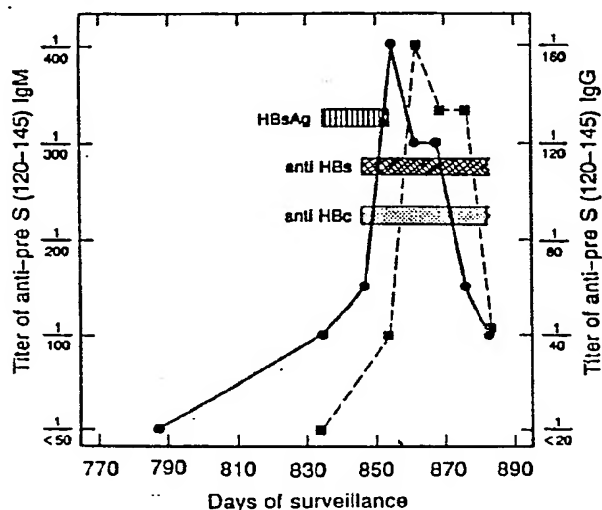
71 Applicant: **New York Blood Center, Inc.,**
310 East 67 Street, New York, New York 10021 (US)
Applicant: **CALIFORNIA INSTITUTE OF TECHNOLOGY,**
1201 East California Boulevard, Pasadena
California 91125 (US)

72 Inventor: **Neurath, Alexander Robert, 230 East 79 Street,**
New York New York 10021 (US)
Inventor: **Kent, Stephen B. H., 615 West California**
Boulevard, Pasadena California 91105 (US)

74 Representative: **Patentanwaltsbüro Cohausz & Florack,**
Postfach 14 01 47, D-4000 Düsseldorf 1 (DE)

54 **Pre-S gene coded peptide hepatitis B immunogens, vaccines, diagnostics, and synthetic lipid vesicle carriers.**

57 A hepatitis B vaccine containing a peptide with an amino acid chain of at least six consecutive amino acids within the pre-S gene coded region of the envelope of hepatitis B virus. The vaccine being free of an amino acid sequence corresponding to the naturally occurring envelope proteins of hepatitis B virus and a physiologically acceptable diluent. The peptide being free or linked to a carrier. The carrier being a conventional carrier or a novel carrier including a lipid vesicle stabilized by cross-linking and having covalently bonded active sites on the outer surface thereon. Such novel carrier being useful not only to link the novel peptide containing an amino acid chain with amino acids within the pre-S gene coded region of the surface antigen of hepatitis B virus, but can also be used to bind synthetic peptide analogues of other viral proteins, as well as bacterial, allergen and parasitic proteins of man and animals. The peptides of the invention can be utilized in diagnostics for the detection of antigens and antibodies.



1
2
3
4
5
6
7 BACKGROUND OF THE INVENTION

8
9 The present invention concerns pre-S gene coded
10 hepatitis B immunogens, vaccines and diagnostics. More
11 especially, this invention concerns novel pre-S gene coded
12 peptides and novel carriers, particularly carriers for pre-S
13 gene coded peptides. Even more especially, the present
14 invention relates to synthetic pre-S gene coded peptides
15 covalently linked to lipid vesicle carriers.

16 There are approximately 600,000 persistent
17 carriers of hepatitis B virus (HBV) in the United States;
18 the estimated total number of carriers in the world is 200
19 million. A considerable portion of HBV carriers have
20 chronic liver disease. The involvement of HBV in liver
21 cancer has been demonstrated (W. Szmuness, Prog. Med. Virol.
22 24, 40 (1978) and R.P. Beasley, L.-Y. Hwang, C.-C. Ling,
23 C.-S. Chien, Lancet Nov., 21, 1129 (1981)).

24 HBV infections thus represent a major public
25 health problem worldwide. Already available vaccines (S.
26 Krugman, in Viral Hepatitis: Laboratory and Clinical
27 Science, F. Deinhardt, J. Deinhardt, Eds., Marcel Dekker,
28 Inc., New York-Basel, 1983, pp. 257-263) produced from the
29 serum of HBV carriers, because of limited resources and
30 production costs involved, do not provide the appropriate

1 means to control and eradicate the disease worldwide. There
2 is hope, however, that this may be accomplished by vaccines
3 based on recombinant DNA technology and/or synthetic
4 peptides.

5 The biology, structure and immunochemistry of HBV
6 and the genetic organization of its DNA genome have been
7 reviewed (B.S. Blumberg, Science, 197 17, (1977)). The
8 cloning and sequencing of the genome of several hepatitis
9 virus (HBV) isolates led to the elucidation of the genetic
10 structure of the viral DNA (P. Tiollais, P. Charnay, G.N.
11 Vyas, Science, 213, 406, (1981)).

12 The immunologic markers of HBV infection include
13 the surface antigen (HBsAg), the core antigen (HBcAg), the
14 "e" antigen (HBeAg) and their respective antibodies.
15 Antibodies against HBsAg are protective against HBV
16 infection.

17 Several antigenic subtypes of HBV and of subvira
18 approximately 22 nm diameter particles (hepatitis B surface
19 antigen; HBsAg) have been recognized (G. Le Bouvier, A.
20 Williams, Am. J. Med. Sci., 270, 165 (1975)). All of these
21 subtypes (for example, ayw, adyw, adw2, adw and adr) share
22 common (group-specific) envelope epitopes, the immune
23 response against which appears sufficient for protection
24 against infection by any of the virus subtypes (W. Szmunes
25 C.E. Stevens, E.J. Harley, E.A. Zang, H.J. Alter, P.E.
26 Taylor, A. DeVera, G.T.S. Chen, A. Kellner, et al., N. Eng
27 J. Med., 307, 1481, (1982)).

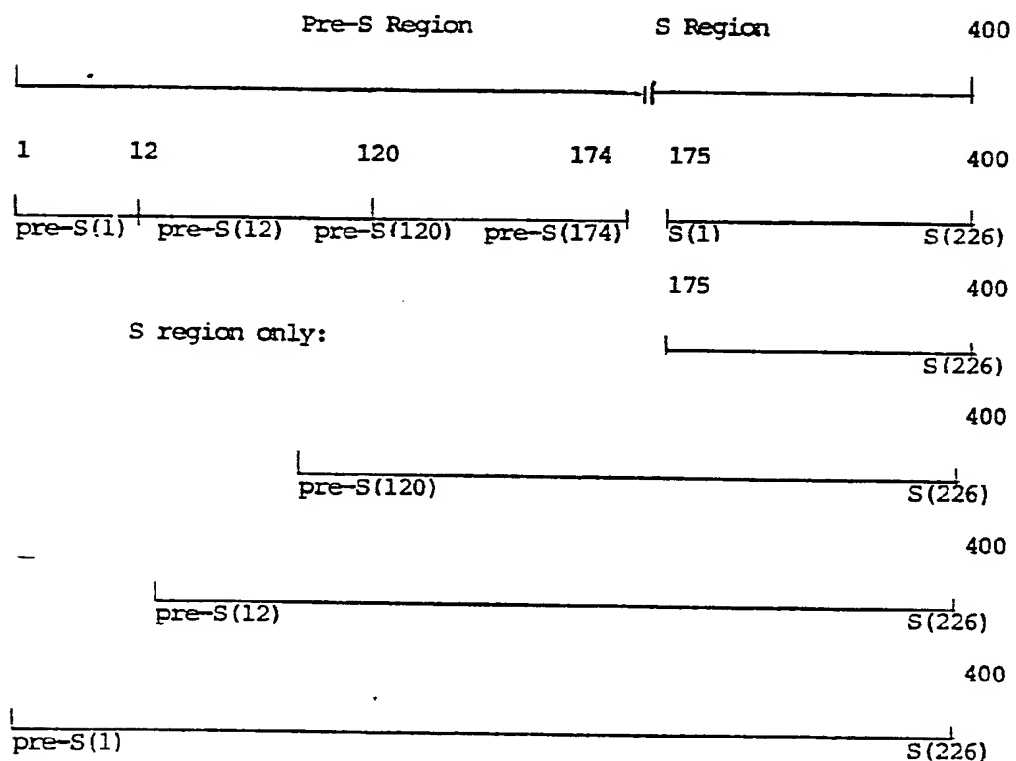
28 The physical structure and proposed genetic
29 organization of the HBV genome are described by Tiollais et
30

0154902

al, 1981, supra at pp 408-409. There are two DNA strands, namely the long (L) strand and the short (S) strand. The L strand transcript has four open reading frame regions which are termed (S + pre-S), C, P and X.

The open reading frame region (S + pre-S) corresponds to the envelope (env) gene of HBV DNA and codes for a family of proteins found in the HBV envelope and in virus related particles.

A schematic representation of the potential translation products of the env gene(s) of HBV DNA is as follows:



The numbers in the above schematic refers to amino acids (AA). A translation initiation site at Met 1 exists

0154902

1 for the adw₂ and adr subtypes only. The first amino acid
2 for the other subtypes correspond to position pre-S 12.

3 Hereinafter, amino acid sequences corresponding to
4 the pre-S region (env 1 to 174) are designated with the
5 prefix "pre-S" and amino acid sequences corresponding to the
6 S region (env 175 to 400) are designated by the prefix "S".
7 In the env gene product representation, the S region spans
8 amino acids 175 to 400 as compared to amino acids 1 to 226
9 in the "S region only" representation.

10 In the above schematic, the pre-S region is
11 defined by amino acid sequence positions pre-S 1 to amino
12 acid sequence position pre-S 174. The S region is defined by
13 sequence positions S 1 (amino acid 175 of the open reading
14 frame and adjacent to pre-S 174) to sequence position S 266
15 (amino acid 400 of the open reading frame). The s-gene
16 product (S-protein) consists of this 226 amino acid
17 sequence.

18 The epitope(s) essential for eliciting
19 virus-neutralizing antibodies have not yet been
20 unambiguously defined. It has been reported that the
21 group-specificity is represented by a complex of
22 determinants located on each of the two major approximately
23 22 and approximately 26 kilodalton constituent proteins (P22
24 and P26) of the virus envelope and of the hepatitis B
25 surface antigen (HBsAg). See J.W.-K. Shih, J.L. Gerin, J.
26 Immunol., 115, 634, (1975); J.W.-K. Shih, P.L. Tan, J.L.
27 Gerin, J. Immunol., 120, 520, (1978); S. Mishiro, M. Imai,
28 K. Takahashi, A. Machida, T. Gotanda, Y. Miyakawa, M.

0154902

1 Mayumi, J. Immunol., 124, 1589, (1980); and G.R. Dreesman,
2 R. Chairez, M. Suarez., F.B. Hollinger, R.J. Courtney, J.L.
3 Melnick, J. Virol., 16, 568, (1975).

4 These proteins have identical amino acid sequences
5 coded for by the S-gene of HBV DNA (Tiollais et al, supra),
6 but the larger protein also carries carbohydrate chains.
7 Peptides corresponding to selected segments of the S-gene
8 product were synthesized and shown to elicit antibodies to
9 HBsAg (anti-HBs). However, immunization of chimpanzees with
10 these peptides resulted in only partial protection against
11 HBV infection (N. Williams, Nature, 306, 427, (1983)).

12 It has been reported recently that the minor
13 glycoprotein components of HBsAg with M_r of approximately 33
14 and approximately 36 kilodaltons (P33, P36) are coded for
15 HBV DNA and contain the sequence of P22 (226 amino acids
16 corresponding to the S region) and have 55 additional amino
17 acids at the amino-terminal part which are coded by the
18 pre-S region of the env gene(s) of HBV DNA. See W. Stibbe,
19 W.H. Gerlich, Virology, 123, 436, (1982); M.A. Feitelson,
20 P.L. Marion, W.S. Robinson, Virology, 130, 76, (1983); W.
21 Stibbe, W.H. Gerlich, J. Virol., 46, 626, (1983); and A.
22 Machida, S. Kishimoto, H. Ohnuma, H. Miyamoto, K. Baba, K.
23 Oda, T. Nakamura, Y. Miyakawa, M. Mayumi, Gastroenterology,
24 85, 268, (1983). Machida et al describe an amino acid
25 sequence composition as a receptor for polymerized serum
26 albumin.

27
28 Heretofore, amino acid sequences coded for by the
29 pre-S region of the hepatitis B virus DNA were virtually
30

1 completely ignored for purposes of producing synthetic
2 vaccines. The hepatitis B vaccine currently in use in the
3 United States lacks the pre-S gene coded sequences (and
4 therefore does not elicit antibodies to such sequences) and
5 thus elicits an immune response to the HBV envelope which is
6 incomplete as compared with that occurring during recovery
7 from natural infection.

8 The generation of antibodies to proteins by
9 immunization with short peptides having the amino acid
10 sequence corresponding to the sequence of preselected
11 protein fragments appears to be a frequent event (Nima,
12 H.L., Houghten, R.A., Walker, L.E., Reisfeld, R.A., Wilson,
13 I.A., Hogle, J.M. and Lerner, R.A., "Generation Of
14 Protein-Reactive Antibodies By Short Peptides Is An Event Of
15 High Frequency: Implications For The Structural Basis Of
16 Immune Recognition", Proceedings of the National Academy of
17 Sciences USA, 80, 4949-4953, (1983)). Nevertheless, the
18 generation of antibodies which recognize the native protein
19 may depend on the appropriate conformation of the synthetic
20 peptide immunogen and on other factors not yet understood.
21 See Pfaff, E., Mussgay, M., Böhm, H.O., Schulz, G.E. and
22 Schaller, H., "Antibodies Against A Preselected Peptide
23 Recognize And Neutralize Foot And Mouth Disease Virus", The
24 EMBO Journal, 7, 869-874, (1982); Neurath, A.R., Kent,
25 S.B.H. and Strick, N., "Specificity Of Antibodies Elicited
26 By A Synthetic Peptide Having A Sequence In Common With A
27
28
29
30

1 Fragment Of A Virus Protein, The Hepatitis B Surface
2 Antigen," Proceedings Of The National Academy Of Sciences
3 USA, 79, 7871-7875, (1982); Ionescu-Matiu, I., Kennedy,
4 R.C., Sparrow, J.T., Culwell, A.R., Sanchez, Y., Melnick,
5 J.L. and Dreesman, G.R., "Epitopes Associated With A
6 Synthetic Hepatitis B Surface Antigen Peptide", The Journal
7 Of Immunology, 130, 1947-1952, (1983); and Kennedy, R.C.,
8 Dreesman, G.R., Sparrow, J.T., Culwell, A.R., Sanchez, Y.,
9 Ionescu-Matiu, I., Hollinger, F.B. and Melnick, J.L. (1983);
10 "Inhibition Of A Common Human Anti-Hepatitis B Surface
11 Antigen Idiotypic By A Cyclic Synthetic Peptide," Journal of
12 Virology, 46, 653-655, (1983). For this reason, immunization
13 with synthetic peptide analogues of various virus proteins
14 has only rarely resulted in production of virus-neutralizing
15 antisera comparable to those elicited by the viruses (virus
16 proteins) themselves (Pfaff et al., 1982, supra). Thus, the
17 preparation of synthetic immunogens optimally mimicking
18 antigenic determinants on intact viruses remains a
19 challenge.
20

21 Replacement of commonly used protein carriers,
22 namely keyhole limpet hemocyanin (KLH), albumin, etc., by
23 synthetic carriers, represents part of such challenge.
24 Although recent reports indicate that free synthetic
25 peptides can be immunogenic, (Dreesman, G.R., Sanchez, Y.,
26 Ionescu-Matiu, I., Sparrow, J.T., Six, H.R., Peterson, D.L.,
27 Hollinger, F.B. and Melnick, J.L., "Antibody To Hepatitis B
28
29
30

peptides can be immunogenic, (Dreesman, G.R., Sanchez, Y., Ionescu-Matiu, I., Sparrow, J.T., Six, H.R., Peterson, D.L., Hollinger, F.B. and Melnick, J.L., "Antibody To Hepatitis B Surface Antigen After A Single Inoculation Of Uncoupled Synthetic HBsAg Peptides" Nature, 295, 158-160, (1982), and Schmitz, H.E., Atassi, H., and Atassi, M.Z., "Production Of Monoclonal Antibodies To Surface Regions That Are Non-Immunogenic In A Protein Using Free Synthetic Peptide As Immunogens: Demonstration With Sperm-whale Myoglobin", Immunological Communications, 12, 161-175, (1983)), even in these cases the antibody response was enhanced by linking of the peptides to a protein carrier (Sanchez, Y., Ionescu-Matiu, I., Sparrow, J.T., Melnick, J.L., Dreesman, G.R., "Immunogenicity Of Conjugates And Micelles Of Synthetic Hepatitis B Surface Antigen Peptides", Intervirology, 18, 209-213, (1982)).

For commonly used protein carriers there is a strong immune response to the carrier, as well as the synthetic peptide. Thus, it would be advantageous to evoke an anti-HBs response with peptides by use of non-protein carriers, which themselves do not evoke an antibody response.

The possible use of several distinct vaccines in prophylaxis would be facilitated by the availability of fully synthetic immunogens.

DEFINITIONSAmino Acid Code Words (as appearing in Fig. 2)

1			
2			
3	D	Asp	aspartic acid
4	N	Asn	asparagine
5	T	Thr	threonine
6	S	Ser	serine
7	E	Glu	glutamic acid
8	Q	Gln	glutamine
9	P	Pro	proline
10	G	Gly	glycine
11	A	Ala	alanine
12	C	Cys	cysteine
13	V	Val	valine
14	M	Met	methionine
15	I	Ile	isoleucine
16	L	Leu	leucine
17	Y	Tyr	tyrosine
18	F	Phe	phenylalanine
19	W	Trp	tryptophane
20	K	Lys	lysine
21	H	His	histidine
22	R	Arg	arginine
23			
24	<u>HBV</u>		hepatitis B virus
25	<u>HBsAg</u>		hepatitis B surface antigen.
26	<u>DNA</u>		deoxyribonucleic acid
27			
28			
29			
30			

SUMMARY OF THE INVENTION

The applicants have found that antibodies to the pre-S protein appear early in the course of hepatitis B infection and probably play the role of antibodies eliminating HBV from the circulation and thus interrupting further spread of the infection. Antibodies to the pre-S protein are likely to represent virus-neutralizing antibodies. The failure of some hepatitis B vaccines to elicit such antibodies may be of considerable biological significance, as indicated by poor immunoprophylactic effects elicited by such vaccines in some populations, despite a detectable immune response to the S-protein.

Applicants have discovered that amino acid sequences coded for by the pre-S region of the env gene of hepatitis B virus (HBV) DNA carry dominant antigenic determinants common to intact and denatured HBsAg. Applicants have found that immuno-dominant disulfide bond-independent epitopes recognized by human antibodies to hepatitis B virus (HBV) exist within proteins containing amino acid sequences coded by the pre-S region of HBV DNA, and more particularly within proteins containing an N-terminal portion (coded for the pre-S region of HBV DNA) having an N-terminal methionine at amino acid sequence position pre-S 120. Applicants further discovered that peptides corresponding to amino acid sequences in the pre-S region, and more particularly in the aforementioned region

1 starting at amino acid 120 of the env gene open reading
2 frame, inhibit the reaction between human anti-HBs and P33
3 (P36), are highly immunogenic, and elicit high levels of
4 group-specific antibodies against HBsAg and HBV. The
5 immunogenicity of such peptides is enhanced by covalent
6 linking to novel lipid vesicle (liposome) carriers also
7 discovered by applicants.

8 Glutaraldehyde-fixed liposomes were found by
9 applicants to be preferred carriers for the peptides of the
10 invention for inducing anti-HBs.

11 The present invention thus concerns a hepatitis B
12 peptide immunogen including a peptide containing an amino
13 acid chain corresponding to at least six consecutive amino
14 acids within the pre-S gene coded region of the envelope of
15 HBV. The hepatitis B peptide immunogen being free of an
16 amino acid chain corresponding to the naturally occurring
17 envelope proteins of hepatitis B virus.

18 The naturally occurring envelope proteins of
19 hepatitis B virus include the following:
20

21 (1) a full length translational product of the
22 env gene of HBV, i.e., for adw₂ and adr pre-S(1-174) +
23 S(175-400)=400 amino acids, for ayw, adyw and adw
24 pre-S(12-174) + S(1-226) = 389 amino acids (env 12-400);

25 (2) pre-S(120-174) + S(175-400) = 281 amino acids
26 (env 120-400) = terminal 55 amino acids in the pre-S region
27
28
29
30

1 + 226 amino acids comprising the entire S region (the
2 corresponding proteins approximately 33 and 36 kD in size
3 (P33 and P36), and differing from each other in the extent
4 of glycosylation); and

5 (3) S(1-226) = 226 amino acids, i.e., the entire
6 S region (env 175-400); representing the approximately 22
7 and 26 kD major constituents of the HBV envelope (P22 and
8 P26) in their non-glycosylated and glycosylated forms (the
9 "S-protein").

10 In an embodiment of the hepatitis B peptide
11 immunogen of the present invention, the corresponding chain
12 of amino acids lies between the sequence positions pre-S 120
13 and pre-S 174. In another embodiment of the invention, the
14 chain of amino acids is between sequence positions pre-S 1
15 and pre-S 120. In a further embodiment of the invention,
16 the corresponding chain of amino acids includes the
17 methionine amino acid at sequence position pre-S 120. In
18 still another embodiment, the chain of amino acids is an
19 amino acid chain containing at least 26 amino acids in the
20 pre-S region. Still further, the chain of amino acids
21 containing at least 26 amino acids can correspond to a chain
22 of at least 26 consecutive amino acids disposed between
23 sequence position pre-S 120 and sequence position pre-S 174.
24 Generally the peptide has no more than 280 amino acids,
25 preferably no more than 225 amino acids, more preferably no
26
27
28
29
30

1 more than 174 amino acids, even more preferably no more than
2 100 amino acids, and still more preferably, no more than 50
3 amino acids. The vaccine of the present invention can be
4 composed solely of a peptide, or preferably of a peptide
5 joined to a carrier. Such carrier can be a conventional
6 carrier, or a novel carrier according to the present
7 invention as described hereinbelow.

8 The hepatitis B peptide immunogen of the present
9 invention is free of any serum proteins, e.g., blood serum
10 proteins.

11 The present invention also concerns a hepatitis B
12 vaccine including a peptide containing an amino acid chain
13 corresponding to at least six consecutive amino acids within
14 the pre-S gene coded region of the envelope of HBV, and a
15 physiologically acceptable diluent, e.g., phosphate buffered
16 saline. The hepatitis B peptide vaccine being free of an
17 amino acid chain corresponding to the naturally occurring
18 envelope proteins of hepatitis B virus.

19 The present invention is also directed to a novel
20 carrier for peptides. In a particularly preferred embodiment
21 of the present invention, the hepatitis B vaccine containing
22 an amino acid chain corresponding to a chain of amino acids
23 in the pre-S region is linked to a carrier via active sites
24 on the carrier. Still more preferred, the carrier is a lipid
25 vesicle carrier. Even more preferred, the lipid vesicle
26 carrier is stabilized by cross-linking.

1 The carrier of the present invention includes a
2 lipid vesicle stabilized by cross-linking and having
3 covalently bonded active sites on the outer surface thereof.
4 The synthetic peptide is bonded via such active sites on the
5 carrier to the outer surface of the lipid vesicle. Such
6 active sites include -COOH , -CHO , -NH_2 and -SH . Such
7 stabilization by cross-linking is accomplished by a
8 stabilizing agent such as an aldehyde having at least two
9 functional groups, such as a bifunctional aldehyde, for
10 example, glutaraldehyde. The carrier of the present
11 invention is chemically cross-linked with pendant functional
12 groups.
13

14 The present application also concerns diagnostic
15 methods. The present invention relates to processes for
16 detecting the presence of either pre-S gene coded
17 hepatitis B antigens or antibodies in a serum.

18 Antibodies to the synthetic peptides disclosed
19 herein can be detected in samples by a process which
20 comprises:

21 a) contacting the sample with a solid substrate
22 coated with a non-labelled peptide containing an amino acid
23 chain corresponding to at least six consecutive amino acids
24 within the pre-S gene coded region of the envelope of HBV,
25 the peptide free of an amino acid sequence corresponding to
26 the naturally occurring envelope proteins of hepatitis B
27 virus, incubating and washing said contacted sample;

28 b) contacting the incubated washed product
29 obtained from step a above with a labelled peptide
30

1 containing an amino acid chain corresponding to at least six
2 consecutive amino acids within the pre-S gene coded region
3 of the envelope of HBV, said peptide free of an amino acid
4 sequence corresponding to the naturally occurring envelope
5 protein of hepatitis B virus, incubating and washing the
6 resultant mass; and

7 c) determining the extent of labelled peptide
8 present in the resultant mass obtained by step b above.

9 Such a process is normally performed using a solid
10 substrate which is substantially free of available protein
11 binding sites. Such as by binding sites unbound by
12 unlabelled peptide with a protein binding site occupier,
13 e.g., albumin.
14

15 Another process for detecting such antibodies
16 comprises:

17 a) contacting the sample with a solid substrate
18 coated with a non-labelled peptide containing an amino acid
19 chain corresponding to at least six consecutive amino acids
20 within the pre-S gene coded region of the envelope of HBV,
21 the peptide free of an amino acid sequence corresponding to
22 the naturally occurring envelope proteins of hepatitis B
23 virus, incubating and washing said contacted sample;

24 b) contacting the incubated washed product
25 obtained from step a above with labelled antibody to human
26 or animal immunoglobulin product by contact with an
27 immunogen comprising a peptide corresponding to at least six
28 consecutive amino acids within the pre-S gene coded region
29 of the envelope of HBV, the peptide immunogen free of an
30 amino acid sequence corresponding to the naturally occurring

1 envelope proteins of hepatitis B virus, incubating and
2 washing the contacted sample, and

3 c) determining the extent of labelled antibody
4 present in the resultant mass of step b.

5 HBV or HBsAg can be detected in a sample by a
6 process which comprises:

7 a) contacting a first portion of a composition
8 containing an antibody produced by introducing into an
9 animal or human an immunogen comprising a peptide
10 corresponding to at least six consecutive amino acids within
11 the pre-S gene coded region of the envelope of HBV, the
12 peptide immunogen free of an amino acid sequence
13 corresponding to the naturally occurring envelope proteins
14 of hepatitis B virus, with a mixture of said sample and the
15 immunogen which has been labelled, incubating and washing
16 the first portion;

17 b) contacting a second portion of the
18 composition containing antibody with the same amount of the
19 labelled immunogen in an antigen free control, incubating
20 and washing the second portion;

21 c) adding the same amount of Staphylococci
22 bearing protein A to each of the compositions of steps a and
23 b above, incubating both of the compositions, centrifuging
24 each of the compositions and separating liquid from the
25 solids therein;

26 d) determining the extent of labelled immunogen
27 in each of the resultant compositions from step c above, and

28 e) comparing the relative amount of labelled
29 immunogen in each such that if the activity of the resultant
30

1 composition containing the first portion is less than the
2 activity for the resultant composition of the second
3 portion, then the sample contains HBV or HBsAg.

4 The synthetic immunogens can be used in general in
5 both sandwich type immunoassays and competition type
6 immunoassays, such as those immunoassays in which antigen in
7 the sample competes with labelled immunogen for antibody.

8 These and other suitable immunoassay schemes for
9 use in connection with the synthetic immunogens of this
10 invention and antibodies thereto are disclosed in copending
11 application Serial No. 426,309, filed September 29, 1982,
12 entitled Labelled Peptides As Diagnostic Reagents, assigned
13 to one of the assignees hereof, the disclosure of which is
14 hereby incorporated herein by reference.

15 The present invention also concerns a diagnostic
16 test kit for detecting hepatitis B virus in sera comprising

17 a) antibodies to a peptide containing an amino
18 acid chain corresponding to at least six consecutive amino
19 acids within the pre-S gene coded region of the envelope of
20 HBV, the peptide being free of an amino acid chain
21 corresponding to the naturally occurring envelope proteins
22 of hepatitis B virus, attached to a solid support,

23 — c) labelled antibodies to the peptide or to
24 hepatitis B virus.

25 The kit can comprise a set of instructions for
26 effecting an immunoassay wherein the effect of formation of
27 an immune complex is revealed by said labelled antibody.

28 The present invention also concerns a diagnostic
29 kit for detecting the presence of antibodies to pre-S gene
30

1 coded antigens of hepatitis B virus in a test sample
2 comprising

3 a) a given amount of a peptide containing
4 an amino acid chain corresponding to at least six
5 consecutive amino acids within the pre-S gene coded region
6 of the envelope of HBV, the peptide being free of an amino
7 acid chain corresponding to the naturally occurring envelope
8 proteins of hepatitis B virus. The peptide is attached to a
9 solid support, e.g., a water insoluble solid support.

10 b) labelled antibodies, e.g., radiolabeled
11 or enzyme labelled, to human IgG and/or IgM.

12 The kit can comprise a set of instructions for
13 effecting an immunoassay, wherein the extent of formation of
14 an immune complex is revealed by said labelled antibodies.

15 In a particular aspect, the present invention
16 concerns a process for the detection of antigens coded for
17 the pre-S gene in sera of HBV infected humans and certain
18 animals, for example, chimpanzees, comprising the following
19 steps:

20 (a) coating a solid substrate with
21 antibodies to a peptide having an amino acid chain
22 corresponding to at least six consecutive amino acids within
23 the pre-S gene of HBV DNA, the peptide being free of an
24 amino acid sequence corresponding to the naturally occurring
25 envelope proteins of HBV,

26 (b) washing the coated substrate;

27 (c) contacting the washed coated substrate,
28 e.g., polystyrene beads; with a protein-containing solution;

29 (d) washing the substrate from step c;
30

0154902

1 (e) incubating the substrate from step d
2 with a sample suspected to contain HBV or HBsAg;

3 (f) washing the substrate from step e;

4 (g) adding radiolabeled or enzyme-labeled
5 antibody, the antibody being an antibody to the peptide or
6 HBsAg;

7 (h) incubating the substrate from step g;

8 (i) washing the substrate from step h; and

9 (j) subjecting the substrate of step i to
10 counting in a gamma counter, or measuring its enzymatic
11 activity.

12 The above process can be conducted using ELISA
13 techniques rather than RIA detection techniques.

14 In a particular embodiment, the present invention
15 also relates to a process for the detection of antibodies to
16 proteins coded for by the pre-S region of hepatitis B virus
17 DNA, comprising the following steps:

18 (a) adsorbing on a solid substrate
19 containing binding sites thereon, e.g., polystyrene beads, a
20 peptide having an amino acid sequence corresponding to at
21 least six consecutive amino acids within the pre-S gene
22 coded region of the HBV envelope, the peptide being free of
23 an amino acid sequence corresponding to the naturally
24 occurring envelope proteins of hepatitis B virus,

25 (b) contacting the substrate from step a
26 with a material to saturate the binding sites thereon,

27 (c) washing the substrate from step b,

28 (d) contacting the substrate from step c
29 with a specimen comprising human sera,
30

- 1 (e) incubating the resultant mass of step d,
- 2 (f) washing the resultant mass of step e,
- 3 (g) adding radiolabeled antibodies to human
- 4 IgG or IgM to the resultant mass of step f to form a second
- 5 resultant mass,
- 6 (h) subjecting the second resultant mass of
- 7 step g to counting in a gamma counter,
- 8 (i) subjecting normal sera utilized as a
- 9 control to steps (a) to (h) and
- 10 (j) comparing the counts of steps h and i.

11 In the above process for the detection of
12 antibodies, ELISA techniques can be substituted for RIA
13 techniques.

14 The present invention also relates to a process
15 for predicting the outcome of hepatitis B infection which
16 comprises carrying out an immunoassay on serum of a human to
17 detect the presence of an antibody to an antigen coded for
18 by the pre-S gene coded region of the envelope of hepatitis
19 B virus employing the above-described hepatitis B peptide
20 immunogen at regular intervals and evaluating the data.

21 The present invention further relates to a process
22 for determining if a human who has been vaccinated with a
23 vaccine against hepatitis B has become immune to hepatitis B
24 virus. Such process involves effecting a plurality of
25 immunoassays of serum from such human to determine if there
26 are antibodies in the serum to an antigen coded by the pre-S
27 gene coded region of the envelope of hepatitis B virus
28 employing the above-described hepatitis B peptide immunogen,
29 the immunoassays being performed on serum taken from the
30

1 human at different times.

2 The present invention further concerns a method
3 for detecting the presence of hepatitis B virus infection
4 comprising effecting quantitative immunoassays on a serum
5 sample taken from a human to determine the amount of
6 antibodies present therein which are antibodies to an
7 antigen coded by the pre-S gene coded region of the envelope
8 of the hepatitis B virus employing the above-described
9 hepatitis B peptide immunogen and comparing the value with a
10 known standard.

11 The present invention further concerns a method
12 for detecting the presence of hepatitis B virus infection
13 comprising effecting quantitative immunoassays on a serum
14 sample taken from a human to determine the amount of
15 antigens coded by the pre-S gene coded region of the
16 envelope of the hepatitis B virus employing the above-
17 described antibodies to the hepatitis B peptide immunogen
18 and comparing the value with a known standard.

19 The present invention also related to a process
20 for raising antibodies which involves introducing into an
21 animal the above-described hepatitis B peptide immunogen.

22 Still further, the present invention concerns a
23 process for synthesizing His and Trp containing peptides
24 which includes the steps of
25

- 26 a. linking a first amino acid containing an
27 alpha-amino protecting group to a resin;
28 b. removal of the alpha-amino protecting group;
29 c. coupling a second amino acid containing an
30 alpha-amino protecting group to the first amino acid;

1 d. repeating steps b and c by coupling further
2 alpha-protected amino acids to produce a desired peptide,
3 wherein at least one of the amino acids is His and wherein
4 at least one of said amino acids is Trp,

5 e. cleaving the peptide from the resin and
6 removing remaining protective groups to said first amino
7 acids;

8 f. substituting a His(ImDNP) for the His;

9 g. substituting a Trp(InFormyl) for the Trp;

10 h. removing the DNP prior to the cleavage and
11 the removing of protective groups, and

12 i. removing the Formyl during the cleavage and
13 the removing of protective groups.

14 The present invention further concerns a
15 prophylactic method of protecting a patient against becoming
16 infected with hepatitis B comprising administering to such
17 patient, e.g., a human, an effective dosage of a vaccine as
18 described hereinabove

19 BRIEF DESCRIPTION OF THE DRAWINGS

20 Fig. 1 shows the results of submitting reduced
21 HBsAg disassociated into its constituent polypeptides to
22 SDS-polyacrylamide gel electrophoresis ("SDS-PAGE") in urea.
23 Panel a shows the separated proteins detected by a silver
24 stain and panel b is a Western blot with human antiserum to
25 hepatitis B.
26 hepatitis B.

27 Fig. 2 shows amino acid sequences of the
28 translational products of the pre-S gene region deduced from
29 sequences of HBV DNA. The sequences are presented in
30 one-letter amino acid code words (such code words are

1 defined in the Definitions herein). Sequences for five
2 distinct HBV subtypes are presented. The 6th bottom line
3 shows amino acid residues common to all five subtypes.

4 Fig. 3 shows a profile of relative hydrophilicity
5 corresponding to the amino acid sequence of the pre-S gene
6 product. Profiles for subtypes other than ayw are similar.
7 The portion of the profile to the right from methionine 175
8 represents the S-gene translation product.

9 Fig. 4 shows two sets of bar graphs for mean
10 antibody responses of rabbits immunized with free pre-S
11 120-145 (Fig. 4A) and with the same peptide linked to
12 cross-linked liposomes containing L-tyrosine-azobenzene
13 -p-arsenate (RAT) groups (Fig. 4B). Anti-HBs (antibodies to
14 HBsAg), cross-hatched columns; anti-pre-S 120-145,
15 diagonally hatched columns. Similar results to Fig. 4B were
16 obtained with liposomes lacking RAT groups, except that
17 responses after six weeks were lower. Columns corresponding
18 to time = 0 represent sera before immunization.



19 Fig. 5 depicts radioimmunoassays with serial
20 dilutions of a serum from a rabbit immunized with pre-S
21 120-145 linked to liposomes. Anti-HBs (antibodies to HBsAg),
22 ; anti-pre-S 120-145, . Counts per minute (cpm)
23 corresponding to distinct dilutions of the pre-immune serum
24 were subtracted from cpm corresponding to dilutions of
25 anti-pre-S 120-145; the difference was plotted. The endpoint
26 titer of the serum (1/163,840) corresponds to its highest
27 dilution at which the cpm were ≥ 2.1 higher than those
28 corresponding to the same dilution of the pre-immune serum.
29
30

Fig. 6 shows the reaction of anti-pre-S 120-145 with P33 and P36 in a Western blot (similar to Fig. 1).

Fig. 7 shows a graph depicting a diagnostic test for hepatitis B antigens based on polystyrene beads coated with anti-pre-S 120-145.

Fig. 8 depicts a plot representing the compilation of antibody responses of individual rabbits to conjugates of S135-155 (amino acids 309 to 329 of the open reading frame of the HBV env gene). The type of conjugates is indicated by numbers defined in Table 1. Antibodies in sera obtained two weeks after the third immunization were assayed using a S135-155- beta-galactosidase conjugate and Pansorbin (Neurath et al., 1982, supra). Their relative titer is given in comparison with antibody levels induced by a S135-155-KLH conjugate. Results of anti-HBs assays by RIA (AUSRIA test, Abbott Laboratories, North Chicago, Illinois) are given in international milliunits (mIU/ml; Neurath et al., 1982 supra). The line corresponds to the calculated linear regression that best fits the set of all data concerning rabbits with an anti-HBs response. The calculated correlation coefficient ($= 0.55$) indicates a poor correlation between anti-HBs and anti-S135-155 responses.

Fig. 9 shows four sets of bar graphs (Fig. 9A, Fig. 9B, Fig. 9C and Fig. 9D) depicting examples of time courses of antibody responses in rabbits immunized with distinct S135-155-conjugates (indicated by numbers in each panel and defined in Table 1). Fig. 9A corresponds to conjugate No. 5; Fig. 9B corresponds to conjugate No. 11; Fig. 9C corresponds to conjugate No. 12 and Fig. 9D

1 corresponds to conjugate No. 19. Anti-HBs (dashed columns)
2 and anti-S-135-155 (black columns) were assayed as described
3 for Fig. 8.

4 Fig. 10 shows four plots (A, B, C and D) which
5 depict the kinetics of antibody responses to peptide
6 pre-S(120-145) (■) and to pre-S protein within
7 approximately 22nm spherical HBsAg particles (▣) elicited
8 by unconjugated peptide pre-S(120-145) (plot A) and by the
9 same peptide linked to cross-linked, cysteine-activated
10 liposomes with attached RAT (L-tyrosine
11 azobenzene-p-arsenate) groups (plot B); and the effect of
12 carrier on anti-peptide antibody titers in sera of rabbits
13 immunized with 4 doses of peptides pre-S(120-145) (plot C)
14 and pre-S(12-32) (plot D) given 2 weeks apart. The carriers
15 for plots C and D were: (1) none; (2) keyhole limpet
16 hemocyanin (KLH); (3) alum; (4) and (5) cross-linked,
17 cysteine-activated liposomes with or without attached RAT
18 groups. Complete and incomplete Freund's adjuvant was used
19 in all cases except (3).

21 Fig. 11 shows two plots for radioimmunoassays of
22 IgG antibodies in serial dilutions of rabbit antisera: to
23 pre-S(120-145) (●); to HBV particles and tubular forms of
24 HBsAg (○), devoid of antibodies to S-protein detectable by
25 RIA and to a fusion protein of chloramphenicol
26 acetyltransferase with the sequences of pre-S protein
27 lacking the 41 C-terminal amino acid residues (□); and of
28 IgG (Δ) and IgM (▲) antibodies in serum of a patient
29 recovered from hepatitis B. The latter serum was drawn
30 before antibodies to the S-protein were detectable. Immulon

1 | 2 Removable strings (Dynatech Laboratories) were coated with
2 | 20 μ g/ml of either free peptide pre-S(120-145) or
3 | pre-S(12-32) and post-coated with gelatin (2.5 mg/ml in 0.1
4 | M Tris, pH 8.8). The conditions for coating and the double
5 | antibody RIA are described in A.R. Neurath, S.B.H. Kent, N.
6 | Strick, Science, 224, 392 (1984) and A.R. Neurath, S.B.H.
7 | Kent, N. Strick, Proc. Natl. Acad. Sci USA, 79, 7871 (1982).

8 | Fig. 12 shows a plot depicting the inhibition of
9 | the reaction of anti-pre-S(120-145) IgG (antiserum diluted
10 | 1:100) with a pre-S(120-145)- β -galactosidase conjugate by;
11 | free peptide pre-S(120-145) [\circ]; by 20 nm spherical HBsAg
12 | particles [Δ] and by HBV particles [\square]. The latter two
13 | preparations contained the same concentration of HBsAg
14 | S-protein as determined by radioimmunoassay (AUSRIA, Abbott
15 | Laboratories).

16 | Fig. 13 depicts a plot of titers of anti-pre-
17 | S(120-145) antibodies versus days of surveillance and
18 | indicates the development of IgM [\bullet] and IgG [\square]
19 | antibodies to the pre-S gene coded protein of HBV during
20 | acute hepatitis B.

21 | Fig. 14 shows a plot for radioimmunoassays of
22 | various preparations containing HBV-specific proteins on
23 | polystyrene beads coated either with anti-pre-S(120-145) I
24 | (\circ , \bullet , \square) or with IgG from a rabbit antiserum against HBV
25 | particles and tubular forms of HBsAg (Δ , \triangle). The tested
26 | antigens were: HBV particles and tubular forms (\bullet , Δ);
27 | approximately 20 nm spherical particles of HBsAg isolated
28 | from plasma (\circ , Δ); and the latter particles treated with
29 |
30 |

0154902

pepsin (1 mg/ml HBsAg, 50 µg/ml pepsin in 0.1 M glycine-HCl, pH 2.2, 2 hours at 37°C) (□).

Fig. 15 depicts a plot for radioimmunoassays of polymerized albumin-binding sites associated with HBsAg isolated from human plasma and containing pre-S gene coded sequences (●) or with HBsAg produced in yeast transfected with recombinant DNA containing the HBV DNA S-gene and thus lacking pre-S gene coded sequences (○).

DETAILED DESCRIPTION OF THE INVENTION

Amino acid sequences deduced from sequences of the pre-S portion of the env genes corresponding to several HBV subtypes (see Fig. 2) have the following properties distinct from those of the S-protein: (i) high hydrophilicity and high percentage of charged residues (E. Schaeffer, J.J. Sninsky, Pro. Natl. Acad. Sci. USA, 81, 2902 (1984)); (ii) absence of cysteine residues; (iii) the highest subtype-dependent variability among HBV DNA gene products; and (iv) little homology with analogous sequences corresponding to nonhuman hepadnaviruses (F. Galibert, T.N. Chan, E. Mandart, J. Virol., 41, 51, (1982)). These properties suggest that the pre-S gene coded portion of the HBV envelope is exposed on the surface of the virion, is a target for the host's immune response and is responsible for the host range of HBV (limited to humans and some primates). Synthetic peptides and antibodies against them, having predetermined specificity offer the opportunity to explore the biological role of the pre-S protein moiety of the HBV envelope.

1 Cleavage of disulfide bonds within HBsAg results
2 in:

3 (a) a substantial decrease of binding of
4 polyclonal antibodies (G.N. Vyas, K.R. Rao, A.B. Ibrahim,
5 Science, 178, 1300, (1972); N. Sukeno, R. Shirachi, J.
6 Yamaguchi, N. Ishida, J. Virol., 9, 182, (1972); G.R.
7 Dreesman, F.B. Hollinger, R.M. McCombs, J.L. Melnick, J.
8 Gen. Virol. 19, 129 (1973); and A.R. Neurath, N. Strick, J.
9 Med. Virol., 6, 309, (1980)) and of some monoclonal
10 antibodies (J. Pillot, M.M. Riottot, C. Geneste, L.
11 Phalente, R. Mangalo, Develop. Biol. Stand., in press
12 (1984)) elicited by intact HBsAg, and

13 (b) reduction of immunogenicity (Y. Sanchez, I.
14 Ionescu-Matiu, J.L. Melnick, G.R. Dreesman, J. Med. Virol.
15 11, 115, (1983)). However, some epitopes are resistant to
16 reduction of disulfide bonds (M. Imai, A. Gotoh, K.
17 Nishioka, S. Kurashina, Y. Miyakawa, M. Mayumi, J. Immunol.,
18 112, 416, (1974)). These epitopes are common to all
19 antigenic subtypes of HBV, but their localization on
20 envelope components of HBV has not been determined. The
21 present invention takes advantage of the localization of
22 disulfide-bond independent antigenic determinants on the
23 N-terminal portion (coded for by the pre-S gene of HBV DNA)
24 of the minor HBsAg proteins P33 and P36, and on other
25 regions of proteins coded for by the pre-S gene.

26 These determinants represent the dominant epitopes
27 on reduced and dissociated HBsAg reacting with human
28 anti-HBs. They are mimicked with high fidelity by pre-S
29 120-145 which elicits antibodies to HBsAg about 400 times
30

1 more efficiently than a synthetic peptide analogue
2 corresponding to the S-gene (A.R. Neurath, S.B.H. Kent, and
3 N. Strick, Proc. Natl. Acad. Sci. USA, 79, 7871 (1982)). No
4 precedent exists for such high levels of virus-recognizing
5 antibodies to a synthetic peptide analogue of an HBV
6 protein. These antibodies could be used in a diagnostic test
7 permitting the direct detection of the pre-S gene coded
8 antigenic determinants in serum of HBV carriers.

9 The pre-S gene is the most divergent among all
10 regions of hepadnavirus genomes (F. Galibert, T.N. Chen, E.
11 Mandart, J. Virol., 41, 51 (1982)) (HBV is a member of the
12 hepadnavirus family).

13 The hepatitis B vaccine of the present invention
14 contains a peptide, either a synthetic peptide (peptide
15 produced by assembling individual amino acids by chemical
16 means or by expression vectors (DNA route)) or a peptide
17 derived from natural sources, such peptide having an amino
18 acid chain corresponding to at least six consecutive amino
19 acids within the pre-S gene coded region of the surface
20 antigen of hepatitis B virus. Such chain can be, for
21 example, at least 10, 15, 20, or 26 amino acids long. A
22 preferred peptide according to one embodiment of the present
23 invention is an amino acid chain disposed between sequence
24 position pre-S 120 and pre-S 174, and more preferably such
25 chain includes the N-terminal methionine at sequence
26 position pre-S 120. A preferred peptide is an amino acid
27 chain corresponding to the chain between sequence position
28 pre-S 120 and pre-S 145, i.e., pre-S (120-145).
29
30

1 Preferred positions of the chain include the
2 following: (1) The amino acid chain entirely between and
3 including sequence position pre-S 1 and pre-S 11 for
4 subtypes adw₂ and adr, (2) between and including sequence
5 positions pre-S 10 and pre-S 40, (3) between and including
6 sequence positions pre-S 15 and pre-S 120, (4) between and
7 including sequence position pre-S 15 and pre-S 55, and (5)
8 between and including sequence position pre-S 90 and pre-S
9 120. A particularly preferred chain according to the
10 present invention has 26 amino acids, includes the
11 N-terminal methionine at sequence position pre-S 120 and is
12 disposed between sequence position pre-S 120 and pre-S 174.

13 Preferred peptides according to the present
14 invention include the following:

- 15 (1) pre-S(12-32), wherein the sequence is (see
16 Fig. 2) MGTNLSVPNPLGFFPDHQLDP for subtype adw₂;
17 (2) pre-S(120-145), wherein the sequence is (see
18 Fig. 2) MQWNSTAFHQTLQDPRVRGLYLPAGG for subtype adw₂;
19 (3) pre-S(32-53), wherein the sequence is (see
20 Fig. 2) PAFGANSNNPDWDFNPVKDDWP for subtype adw₂;
21 (4) pre-S(117-134), wherein the sequence is (see
22 Fig. 2) PQAMQWNSTAFHQTLQDP for subtype adw₂;
23 (5) pre-S(94-117), wherein the sequence is (see
24 Fig. 2) PASTNRQSGRQPTPISPPLRDSHP for subtype adw₂;
25 (6) pre-S(153-171), wherein the sequence is (see
26 Fig. 2) PAPNIASHISSISARTGDP for subtype adw₂;
27 (7) pre-S(1-21), wherein the sequence is (see
28 Fig. 2) MGGWSSKPRKGMGTNLSVPPNP for subtype adw₂;
29
30

- 1 (8) pre-S(57-73), wherein the sequence is (see
2 Fig. 2) QVGVGAFGPRLTPPHGG for subtype adw₂;
3 (9) pre-S(1-11),
4 a. for adw₂, wherein the sequence is (see
5 Fig. 2) MGGWSSKPRKG
6 b. for adr, wherein the sequence is (see
7 Fig. 2) MGGWSSKPRQG.

8 Any analogs of the pre-S gene coded sequences of
9 the present invention involving amino acid deletions, amino
10 acid replacements, such as replacements by other amino
11 acids, or by isosteres (modified amino acids that bear close
12 structural and spatial similarity to protein amino acids),
13 amino acid additions, or isosteres additions can be
14 utilized, so long as the sequences elicit antibodies
15 recognizing the pre-S protein of HBV or hepatitis B surface
16 antigen.

17 In the formation of a peptide derived from natural
18 sources, a protein containing the required amino acid
19 sequence is subjected to selective proteolysis such as by
20 splitting the protein with chemical reagents or using
21 enzymes. Synthetic formation of the peptide requires
22 chemically synthesizing the required chain of amino acids.

23 — In forming a synthetic vaccine according to the
24 present invention, it is preferred to insure that the amino
25 acid chain (peptide residue) corresponding to at least six
26 consecutive amino acids within the pre-S gene coded region
27 of hepatitis B virus has the steric configuration to be
28 recognized by antibody to hepatitis B virus. To this end,
29 the given chain of amino acids may have bonded thereto as
30

1 part of the amino acid chain, one or more additional amino
2 acids on either, or both sides thereof. These additional
3 amino acids can serve as auxiliary amino acids to enhance
4 the stabilization of the amino acid chain so that it is
5 readily recognized by antibody to hepatitis B virus. The
6 additional amino acids can be the same amino acids in the
7 same sequence as they occur in the natural protein, or other
8 amino acids may be employed.

9 In one form of the invention, the peptide having a
10 chain length of minimally six amino acids can be bounded on
11 either side thereof with additional amino acids, e.g., three
12 amino acids on either side of the residue, to form a longer
13 chain of amino acids. The chain of amino acids may contain
14 more than one amino acid sequence corresponding to at least
15 six consecutive amino acids within the pre-S region of the
16 surface antigen of hepatitis B virus.

17 The length of the individual amino acid sequence
18 would depend on the method of producing the sequence. If
19 the sequence is made by assembling individual amino acids by
20 chemical means, then the sequence length would generally not
21 exceed 50 amino acids, and preferably would not exceed 40
22 amino acids. If the synthetic peptide is obtained from a
23 DNA route, the chain length could be longer, for example,
24 100 or more amino acids. It is, however, normally shorter,
25 and optimally considerably shorter than the natural pre-S
26 protein. Thus, in the embodiment wherein the peptide has
27 units of both the S region and pre-S region, its peptide
28 portions corresponding to the S region is shorter than the
29 natural S protein, e.g., no more than 100 amino acids,
30

1 preferably no more than 40 amino acids and usually less than
2 30 amino acids. In such cases, the peptide portion
3 corresponding to the pre-S region can be of a length
4 corresponding to the entire pre-S region, but generally is
5 less than the entire pre S region.

6 When the peptide contains no components
7 corresponding to the amino acid sequence of the S region, it
8 can contain amino acid sequences corresponding to the entire
9 pre-S region, or shorter than the entire pre-S region.

10 Where, however, the amino acid sequence is part of
11 a long chain, such as when there are more than one sequence
12 of amino acids, the chain can contain residues of various
13 moieties, for example, segments of polyamino acids or
14 polysaccharides.

15 In addition to containing one or more different or
16 the same sequences of amino acids corresponding to at least
17 six consecutive amino acids within the pre-S region of
18 hepatitis B virus, e.g., containing more than one sequence
19 of amino acids corresponding to different epitopes
20 (antigenic determinants) in the pre-S region of hepatitis B
21 virus, the vaccine of the present invention can contain
22 amino acid chains containing epitopes of different antigens
23 or allergens so as to form a vaccine directed to hepatitis B
24 virus and to one or more additional diseases, e.g.,
25 measles, influenza, smallpox, polio, diptheria, just to name
26 a few. Such additional amino acid sequences can be of
27 varying amino acid chain lengths.

28 A hepatitis B vaccine according to the present
29 invention can include in addition to one or more amino acid
30

0154902

sequences corresponding to at least six consecutive amino acids within the pre-S region of the surface antigen of hepatitis B virus, one or more amino acid sequences corresponding to consecutive amino acids within the S region of the surface antigen of hepatitis B virus, for example,

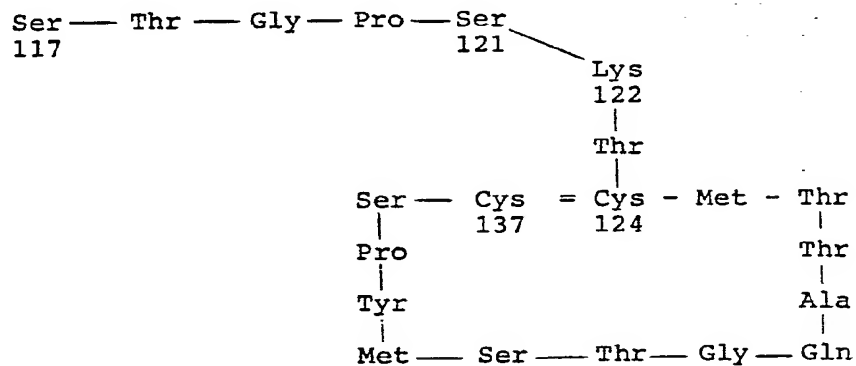
141 142 143 144 145 146
Lys Pro Thr Asp Gly Asn,

or

138 139 140 141 142 143 144 145 146 147 148 149
Cys Cys Thr Lys Pro Thr Asp Gly Asn Cys Thr Cys

Other peptides corresponding to antigenic determinants of HBsAg (S region) and thus combinable in the same chain with one or more amino acids sequences corresponding to at least six amino acids in the pre-S region of HBsAg include the following:

(1)



(2)

Position	Amino Acid Series
48-81	Cys-Leu-Gly-Gln-Asn-Ser-Gln-Ser-Pro-Thr-Ser-Asn-His-Ser-Pro-Thr-Ser-Cys-Pro-Pro-Thr-Cys-Pro-Gly-Thr-Arg-Trp-Met-Cys-Leu-Arg-Arg-Phe-Ile

- 1 (3, 2-16 Glu-Asn-Ile-Thr-Ser-Gly-Phe-Leu-Gly-Pro-
2 Leu-Leu-Val-Leu-Gln-Cys
3
4 (4) 22-35 Leu-Thr-Arg-Ile-Leu-Thr-Ile-Pro-Gln-Ser-
5 Leu-Asp-Ser-Trp-Cys
6
7 (5) 38-52 Ser-Leu-Asn-Phe-Leu-Gly-Gly-Thr-Thr-Val-
8 Cys-Leu-Gly-Gln-Asn
9
10 (6) 47-52 Val-Cys-Leu-Gly-Gln-Asn
11
12 (7) 95-109 Leu-Val-Leu-Leu-Asp-Tyr-Gln-Gly-Met-Leu-
13 Pro-Val-Cys-Pro-Leu
14 (8) 104-109 Leu-Pro-Val-Cys-Pro-Leu
15

16 The sequences of amino acids can be interconnected
17 with one another such as by cross-linking or by being bonded
18 directly thereto in the form of a branched chain, or the
19 respective sequences can be bonded to a central "carrier".

20 There is realized by the present invention a
21 synthetic vaccine which is characterized by the absence of
22 naturally occurring envelope proteins of hepatitis B virus,
23 i.e., the vaccine of the present invention is composed of
24 one or more peptide sequences corresponding to a limited
25 — portion of the hepatitis B virus envelope protein. The
26 vaccine of the present invention is also free of other
27 proteins found in the virion. Vaccines can be synthesized
28 which are free of biologically produced components, free of
29 viral components whether they be active or inactive, free of
30 antibodies, free of deoxyribonucleic acid (DNA), and are

1 therefore likely to be substantially free from undesirable
2 side effects commonly found with other vaccines (e.g.,
3 unintentional infection with virus, allergic reactions,
4 fevers, etc.).

5 It should be understood that the vaccine of the
6 present invention can be in admixture with other proteins
7 and these proteins include the proteins of known antigens or
8 allergens. Thus when it is stated herein that the vaccine
9 is characterized by the absence of an amino acid sequence
10 corresponding to the naturally occurring envelope proteins
11 of the hepatitis B virus it is meant that notwithstanding
12 the absence of such proteins, the composition functions as a
13 vaccine, i.e., provides protective immunization by formation
14 of antibodies.

15 The peptide of the present invention is such that
16 it is capable of forming "neutralizing antibodies", i.e.,
17 antibodies that will protect patients against hepatitis B
18 virus. Accordingly, the present invention is also directed
19 to methods for protecting a patient against contracting
20 hepatitis B.

21 The peptides and vaccines of the present invention
22 can be used to improve immune response and to overcome
23 non-responsiveness to certain known hepatitis B virus
24 vaccines (e.g., containing no peptides corresponding to
25 amino acid sequences in the pre-S region).

26 The peptides of the present invention can be
27 utilized in conjunction with peptides containing amino acid
28 chains corresponding to consecutive amino acids within the S
29 gene coded region of HBsAg. Also, embodied by the present
30

1 invention is a peptide containing amino acids corresponding
2 to consecutive amino acids spanning both the pre-S and S
3 region, e.g., pre-S 160 to S 20.

4 A carrier may be provided for the synthetic
5 peptide of the invention. It should be understood, however,
6 that a carrier may not be required to practice the present
7 invention, i.e., a carrier may not be required to produce
8 antibodies according to the present invention.

9 The "carrier" is simply a physiologically
10 acceptable mass to which the synthetic peptide is attached
11 and which is expected to enhance the immune response. A
12 carrier can comprise simply a chain of amino acids or other
13 moieties and to that end it is specifically contemplated to
14 use as a carrier a dimer, oligomer, or higher molecular
15 weight polymer of a sequence of amino acids defining a
16 synthetic peptide of the invention. In other words, having
17 determined the desired sequence of amino acids to form the
18 synthetic peptide, these amino acids can be formed from
19 naturally available materials or synthetically and can be
20 polymerized to build up a chain of two or more repeating
21 units so that repeating sequences serve both as "carrier"
22 and synthetic peptide. Stated differently, an independent
23 carrier may not be required. Alternatively, additional amino
24 acids can be added to one or both ends of the amino acid
25 chain that defines the synthetic peptide. It is preferred
26 that alternative carriers comprise some substance, animal,
27 vegetable or mineral, which is physiologically acceptable
28 and functions to present the synthetic peptide so that it is
29 recognized by the immune system of a host and stimulates a
30

1 satisfactory immunological response. Thus, a wide variety of
2 carriers are contemplated, and these include materials which
3 are inert, which have biological activity and/or promote an
4 immunological response. For instance, proteins can be used
5 as carriers. Examples of protein carriers include tetanus
6 toxoid, keyhole limpet hemocyanin, etc.

7 Polysaccharides are also contemplated as carriers,
8 and these include especially those of molecular weight
9 10,000 to 1,000,000, including, in particular, starches,
10 dextran, agarose, ficoll or its carboxy methyl derivative
11 and carboxy methyl cellulose.

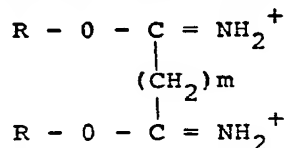
12 Polyamino acids are also contemplated for use as
13 carriers, and these polyamino acids include, among others,
14 polylysine, polyalanyl polylysine, polyglutamic acid,
15 polyaspartic acid and poly (C_2 - C_{10}) amino acids.

16 Organic polymers can be used as carriers, and
17 these polymers include, for example, polymers and copolymers
18 of amines, amides, olefins, vinyls, esters, acetals,
19 polyamides, carbonates and ethers and the like. Generally
20 speaking, the molecular weight of these polymers will vary
21 dramatically. The polymers can have from two repeating units
22 up to several thousand, e.g., two thousand repeating units.
23 Of course, the number of repeating units will be consistent
24 with the use of the vaccine in a host animal. Generally
25 speaking, such polymers will have a lower molecular weight,
26 say between 10,000 and 100,000 (the molecular weight being
27 determined by ultracentrifugation).

28 Inorganic polymers can also be employed. These
29 inorganic polymers can be inorganic polymers containing
30

1 organi. moieties. In particular, silicates and aluminum
 2 hydroxide can be used as carriers. It is preferred that the
 3 carrier be one which is an immunological adjuvant. In such
 4 cases, it is particularly contemplated that the adjuvant be
 5 muramyl dipeptide or its analogs.

6 The carrier can also be the residue of a
 7 crosslinking agent employed to interconnect a plurality of
 8 synthetic peptide containing chains. Crosslinking agents
 9 which have as their functional group an aldehyde (such as
 10 glutaraldehyde), carboxyl, amine, amido, imido or
 11 azidophenyl group. In particular, there is contemplated
 12 the use of butyraldehyde as a crosslinking agent, a divalent
 13 imido ester or a carbodiimide. Particularly contemplated
 14 divalent imido esters are those of the formula



15
 16
 17
 18 wherein m is 1 to 13 and R is an alkyl group of 1 to 4
 19 carbon atoms. Particularly contemplated carbodiimides for
 20 use as crosslinking agents include cyclohexylcarboxiimide,
 21 ethyldimethylaminopropyl carbodiimide, N-ethylmorpholino
 22 cyclohexyl carbodiimide and diisopropyl carbodiimide.

23
 24 Chemical synthesis of peptides is described in the
 25 following publications: S.B.H. Kent, Biomedical Polymers,
 26 eds. Goldberg, E.P. and Nakajima, A. (Academic Press, New
 27 York), 213-242, (1980); A.R. Mitchell, S.B.H. Kent, M.
 28 Engelhard, and R.B. Merrifield, J. Org. Chem., 43,
 29 2845-2852, (1978); J.P. Tam, T.-W. Wong, M. Riemen, F.-S.
 30 Tjoeng, and R.B. Merrifield, Tet. Letters, 4033-4036,

1 (1979); S. Mojsov, A.R. Mitchell, and R.B. Merrifield, J.
2 Org. Chem., 45, 555-560, (1980); J.P. Tam, R.D. DiMarchi and
3 R.B. Merrifield, Tet. Letters, 2851-2854, (1981); and S.B.H.
4 Kent, M. Riemen, M. Le Doux and R.B. Merrifield, Proceedings
5 of the IV International Symposium on Methods of Protein
6 Sequence Analysis, (Brookhaven Press, Brookhaven, N.Y.), in
7 press, 1981.

8 Chemical Synthesis: In the so-called "Merrifield
9 solid phase procedure" the appropriate sequence of L-amino
10 acids is built up from the carboxyl terminal amino acid to
11 the amino terminal amino acid. Starting with the appropriate
12 carboxyl terminal amino acid attached to a polystyrene (or
13 other appropriate) resin via chemical linkage to a
14 chloromethyl group, benzhydrylamine group, or other reactive
15 group of the resin, amino acids are added one by one using
16 the following procedure. The peptide-resin is:
17

18 (a) washed with methylene
19 chloride;

20 (b) neutralized by mixing for 10 minutes at room
21 temperature with 5% (v/v) diisopropyl-
22 ethylamine (or other hindered base) in
23 methylene chloride;

24 (c) washed with methylene chloride;

25 (d) an amount of amino acid equal to six times the
26 molar amount of the growing peptide chain is
27 activated by combining it with one-half as
28 many moles of a carbodiimide (e.g.,
29 dicyclohexylcarbodiimide, or diisopropyl-
30 carbodiimide) for ten minutes at 0°C, to

1 form the symmetric anhydride of the amino
2 acid. The amino acid used should be
3 provided originally as the N-alpha-tert.butyl-
4 oxycarbonyl derivative, with side chains
5 protected with benzyl esters (e.g. aspartic or
6 glutamic acids), benzyl ethers (e.g., serine,
7 threonine, cysteine or tyrosine),
8 benzyloxycarbonyl groups (e.g., lysine) or other
9 protecting groups commonly used in peptide
10 synthesis.

- 11 (e) the activated amino acid is reacted with
12 the peptide-resin for two hours at
13 room temperature, resulting in addition
14 of the new amino acid to the end of the
15 growing peptide chain.
16 (f) the peptide-resin is washed with methylene
17 chloride;
18 (g) the N-alpha-(tert. butyloxycarbonyl) group is
19 removed from the most recently added
20 amino acid by reacting with 30 to 65%, preferably
21 50% (v/v) trifluoroacetic acid in methylene
22 chloride for 10 to 30 minutes at room
23 temperature;
24 (h) the peptide-resin is washed with methylene
25 chloride;
26 (i) steps (a) through (h) are repeated until the
27 required peptide sequence has been
28 constructed.
29

30 The peptide is then removed from the resin and

1 simultaneously the side-chain protecting groups are removed,
2 by reaction with anhydrous hydrofluoric acid containing 10%
3 v/v of anisole or other suitable (aromatic) scavenger.
4 Subsequently, the peptide can be purified by gel filtration,
5 ion exchange, high pressure liquid chromatography, or other
6 suitable means.

7 In some cases, chemical synthesis can be carried
8 out without the solid phase resin, in which case the
9 synthetic reactions are performed entirely in solution. The
10 reactions are similar and well known in the art, and the
11 final product is essentially identical.

12 Isolation from natural sources: If sufficient
13 quantities of the whole protein antigen are available, a
14 limited portion of the molecule, bearing the desired
15 sequence of amino acids may be excised by any of the
16 following procedures:
17

- 18 (a) Digestion of the protein by proteolytic
19 enzymes, especially those enzymes whose
20 substrate specificity results in cleavage
21 of the protein at sites immediately
22 adjacent to the desired sequence of amino
23 acids;
24
25 (b) Cleavage of the protein by chemical means.
26 Particular bonds between amino acids can be
27 cleaved by reaction with specific reagents.
28 Examples include: bonds involving
29 methionine are cleaved by cyanogen bromide;
30 asparaginyl-glycine bonds are cleaved by

1 hydroxylamine;

2 (c) A combination of proteolytic and chemical
3 cleavages.

4 It should also be possible to clone a small
5 portion of the DNA, either from natural sources or prepared
6 by synthetic procedures, or by methods involving a
7 combination thereof, that codes for the desired sequence of
8 amino acids, resulting in the production of the peptide by
9 bacteria, or other cells.

10 Analogously, one can form chains containing a
11 plurality of amino acid sequences by the following
12 technique: An aqueous solution of a peptide or peptides is
13 mixed with a water-soluble carbodiimide (e.g., ethyl-
14 dimethyl-aminopropylcarbodiimide). This results in
15 polymerization of the peptide(s); depending on the use of
16 the side chain blocking groups mentioned above, either
17 straight chain or branched polymers of the peptide can be
18 made.

19 If desired the synthetic peptide of the present
20 invention can have bonded thereto a chain of any of the
21 following moieties: polypeptide, polyamino acid, poly-
22 saccharide, polyamide or polyacrylamide which can serve as a
23 stabilizing chain or as a bridge between amino acids of the
24 individual chains. Such chains are available commercially
25 or, in the case of polyamino acids, are formed by a process
26 which comprises: mixing a solution of the desired amino acid
27 sequence with a solution of the N-carboxylanhydride of the
28 amino acid and allowing a base-catalyzed polymerization to
29
30

1 occur, which is initiated by the amine groups of the
2 peptide.

3 Although a carrier may not be required, if a
4 carrier is employed the deposition of a chain or chains on a
5 "carrier" can be effected as follows:

6 1. Protein Carrier: The protein and the
7 synthetic peptide are dissolved together in water or other
8 suitable solvent, and covalently linked via amide bonds
9 formed through the action of a carbodiimide. The resulting
10 product may contain one or more copies of the peptide per
11 protein monomer. Alternatively, the reduced peptide may be
12 added to a carrier containing sulfhydryl groups to form
13 disulfide bonds. Yet another method involves the addition of
14 reduced peptide to protein carriers containing maleimidyl
15 groups to form a covalent linkage by a Michael addition, or
16 any other covalent attachment means.

17 2. Polysaccharide Carriers: Oligosaccharide
18 carriers should have molecular weights in the range 1,000 to
19 1,000,000. In order to covalently link these to synthetic
20 peptides, suitable functional groups must first be attached
21 to them. Carboxyl groups may be introduced by reacting with
22 iodoacetic acid to yield carboxymethylated polysaccharides,
23 or by reacting with carbonyldiimidazole to yield activated
24 carbonyl esters. Carboxymethyl polysaccharides are coupled
25 to the peptide by a carbodimide reaction, while the
26 activated carbonyl esters react spontaneously with peptides.
27 Multiple copies of the synthetic peptide should be attached
28 to each oligosaccharide unit.

29
30

1 3. Polyamino Acid Carriers: These carriers
2 should have molecular weights in the range 1,000 to
3 1,000,000. Polylysine and polyornithine have primary amino
4 groups on their side chains; polyaspartic acid and
5 polyglutamic acid have carboxyl groups. Peptides may be
6 coupled to these via amide bonds using the carbodiimide
7 reaction. Another carrier that provides amino groups for
8 coupling is polylysine to which polyalanine can be attached
9 to the side chains of the lysine residues. The synthetic
10 peptide may be attached to the ends of polyalanine chains,
11 also by a carbodiimide reaction. Multiple copies of the
12 synthetic peptide should be attached to each oligopep-
13 tide unit.

14 The novel carrier of the present invention
15 includes a lipid vesicle having active sites on the outer
16 surface thereof. Such active sites include -COOH , -CHO ,
17 -NH_2 and -SH . The lipid carrier can be stabilized by
18 cross-linking by a stabilizing agent such as an aldehyde
19 having at least two functional groups, such as a
20 bifunctional aldehyde, e.g., glutaraldehyde.

21 The bonding of the peptide to the lipid vesicle
22 carrier occurs at the active sites on the lipid vesicle on
23 the exterior surface of the carrier. Without wishing to be
24 bound by any theory of operability, it is believed that such
25 bonding is at least covalent bonding.

26 It is possible to bind a peptide to two active
27 sites on the outer surface of the lipid vesicle. For
28 example, a -NH_2 group at one end of a peptide can bind with
29 a -COOH active site on the outer surface of the lipid
30

0154902

1 vesicle. The other end of the peptide can then bind with
2 another active site on the lipid vesicle, for example, a
3 -COOH group on the other end of the peptide can bind with a
4 -NH₂ active site on the lipid vesicle.

5 The preferred carrier to support the synthetic
6 peptides of the present invention is a lipid vesicle. Lipid
7 vesicles can be formed by sonicating a lipid in an aqueous
8 medium, by resuspension of dried lipid layers in a buffer or
9 by dialysis of lipids dissolved in an organic solvent
10 against a buffer of choice. The latter procedure is
11 preferred. Lipid vesicles consist of spheres of lipid
12 bilayers that enclose part of the aqueous medium.

13 Lipid vesicle (non-protein) carriers according to
14 the present invention can be produced in a variety of ways.
15 The preferred method to produce such carriers would be to
16 treat a lipid vesicle containing aminoalkanes and
17 diaminoalkanes having 10 to 18 carbon atoms, for example
18 stearylamine, cetylamine and myrististylamine with a
19 polyaldehyde, such as a dialdehyde, for example, butanedial
20 (succinaldehyde), pentanedial (glutaraldehyde), hexanedial
21 (adipoldehyde), heptanedial (pimelicaldehyde) and octanedial
22 (suberaldehyde). Alternatively, a liposome containing
23 aminoalkenes and diaminoalkenes having 10 to 18 carbon
24 atoms, for example, oleylamine, can be treated with the
25 aforementioned polyaldehydes. The lipid vesicle carrier
26 thus formed has active aldehyde groups on the surface
27 thereof allowing the direct linking of peptides via their
28 N-terminal or lysine groups.
29

30

1 Peptides linked to lipid vesicle carriers
2 ,according to the present invention can also be prepared by
3 treating an amino containing lipid vesicle as described
4 above with a peptide activated by carbodiimide, for example,
5 N-ethyl-N' (dimethylamino)propyl carbodiimide.

6 Alternatively a carbodiimide activated peptide is
7 linked to polyaldehyde, e.g., dialdehyde, treated lipid
8 vesicles which have been further derivatized by reaction
9 with a water-soluble diaminoalkane, e.g., ethylene diamine
10 and propylene diamine.

11 Still further, lipid vesicles containing fatty
12 acids (saturated and unsaturated) having 12 to 18 carbon
13 atoms, e.g., stearic acid, oleic acid, palmitic acid and
14 myristic acid, are activated with carbodiimide. Thereafter,
15 the activated lipid vesicle is reacted with a peptide.

16 Another approach to form a carrier according to
17 the present invention involves using a fatty acid aldehyde
18 as a component of the lipid vesicle and treating such lipid
19 vesicle as described for glutaraldehyde treated lipid
20 vesicles. Such lipid vesicle reacts directly with amino
21 groups of peptides.

22 In a preferred embodiment of a carrier according
23 to the present invention, the aforementioned lipid vesicle
24 carrier formed by treating a amino or diaminoalkane (or
25 amino or diaminoalkene) having 10 to 18 carbon atoms with a
26 polyaldehyde is further reacted with cysteine (L-or D- or
27 LD- cysteine). These lipid vesicles are then reacted with a
28 peptide having -SH groups, i.e., cysteine containing
29
30

0154902

1 peptides. The link between the lipid vesicle and the
2 peptide is mediated by a disulfide bond.

3 Alternatively, a fatty acid mercaptan is used as a
4 component of the lipid vesicle, for example,
5 octadecanethiol. A cysteine containing peptide is directly
6 linked to such lipid vesicle.

7 Another approach to form carriers according to the
8 present invention involves the preparation of the above
9 described fatty acid mercaptan containing lipid vesicles
10 which are further reacted with a dimaleimide, for example,
11 para or ortho N-N'-phenylenedimaleimide. Such lipid vesicle
12 is then reacted with a cysteine containing peptide.

13 Alternatively, the link between the appropriate
14 lipid vesicle and the appropriate peptide can be
15 accomplished by commercially available cross-linking
16 reagents such as dimethyl adipimidate; dimethyl
17 3,3'-dithiobis-propionimidate; 2-iminothiolane;
18 di-succinimidyl suberate; bis[2-(succinimidooxy
19 carbonyloxy)-ethyl] sulfone; disuccinimidyl tartarate;
20 dithiobis (succinimidyl propionate); ethylene glycol
21 bis(succinimidyl succinate); N-5-azido-2-nitrobenzoyloxy-
22 succinimide; p-azidophenacyl bromide; p-azido-phenylglyoxal
23 4-fluoro-3-nitrophenyl azide; N-hydroxysuccinimidyl-4-azide-
24 benzoate; N-hydroxysuccinimidyl-4-azidosalicylic acid; m-
25 maleimidobenzoyl N-hydroxy succinimide ester; methyl-4-
26 azidobenzoimidate; p-nitrophenyl 2-diazo-3,3,3-trifluoro-
27 proprionate; N-succinimidyl-6 (4'-azido-2'-nitrophenylamino
28 nexanoate; succinimidyl 4-(N-maleimidomethyl) cyclohexane-
29 1-carboxylate; succinimidyl 4-(p-maleimidomethyl) butyrate;
30

1 N-(4-azidophenylthio)phthalimide; ethyl 4-aziodophenyl 1,
2 4-dithiobutyrimidate; N-succinimidyl (4-azidophenyldithio)
3 propionate; 1-5-difluoro-2, 4-dinitrobenzene;
4 4,4'-difluoro-3,3'-dinitrodiphenyl-sulfone;
5 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene;
6 p-phenylenediisothiocyanate; 4,4'-dithiobisphenylazide;
7 erythritolbiscarbonate; N-succinimidyl 3-(2-pyridyldithiol)
8 propionate; dimethyl pimelimide and dimethyl suberimide.

9 The lipid vesicles according to the present
10 invention act not only as carriers, but also as adjuvants.

11 The lipid vesicle synthetic carriers of the
12 present invention can be utilized to bind synthetic peptide
13 analogues (eliciting protective antibodies) of various
14 viral, bacterial, allergen and parasitic proteins of man and
15 animals, besides synthetic peptide analogues of hepatitis B
16 surface antigen, and especially the novel synthetic peptide
17 analogue of hepatitis B surface antigen containing amino
18 acid sequences corresponding to amino acid sequences in
19 pre-S gene coded region of the HBV.
20

21 Accordingly, the lipid vesicle synthetic carriers
22 of the present invention can be used to bind with synthetic
23 peptide analogues of the following viruses: influenza
24 hemagglutinin (A/memphis/102/72 strain, A/Eng 1878/69
25 strain, A/NT/60/68/29c strain, and A/Qu/7/70 strain), fowl
26 plague virus hemagglutinin, vaccinia, polio, rubella,
27 cytomegalovirus, small pox, herpes simplex types I and II,
28 yellow fever, Infectious ectromelia virus, Cowpox virus,
29 Infectious bovine rhinotracheitis virus, Equine rhino-
30 pneumonitis (equine abortion) virus, Malignant catarrh virus

0154902

1 of cattle, Feline rhinotracheitis virus, Canine herpes
2 virus, Epstein-Barr virus (associated with infectious
3 mononucleosis and Burkitt lymphoma), Marek's disease virus,
4 Sheep pulmonary adenomatosis (Jaagziekte) virus,
5 Cytomegaloviruses, Adenovirus group, Human papilloma virus,
6 Feline panleucopaenia virus, Mink enteritis virus, African
7 horse sickness virus (9 serotypes), Blue tongue virus (12
8 serotypes), Infectious pancreatic necrosis virus of trout,
9 Fowl sarcoma virus (various strains), Avian leukosis virus
10 (visceral, erythroblastic and myeloblastic), Osteopetrosis
11 virus, Newcastle disease virus, Parainfluenza virus 1,
12 Parainfluenza virus 2, Parainfluenza virus 3, Parainfluenza
13 4, Mumps virus, Turkey virus, CANADA/58, Canine distemper
14 virus, Measles virus, Respiratory syncytial virus,
15 Myxovirus, Type A viruses such as Human influenza viruses,
16 e.g., Ao/PR8/34, A1/CAM/46, and A2/Singapore/1/57; Fowl
17 plaque virus; Type B influenza viruses, e.g., B/Lee/40;
18 Rabies virus; Eastern equine encephalitis virus;
19 Venezuelan equine encephalitis virus; Western equine
20 encephalitis virus; Yellow fever virus, Dengue type 1 virus
21 (=type 6), Dengue type 2 virus (=type 5); Dengue type 3
22 virus; Dengue type 4 virus; Japanese encephalitis virus,
23 Kyasanur Forest virus; Louping ill virus; Murray Valley
24 encephalitis virus; Omsk haemorrhagic fever virus (types I
25 and II); St. Louis encephalitis virus; Human rhinoviruses,
26 Foot-and-mouth disease virus; Poliovirus type 1; Enterovirus
27 Polio 2; Enterovirus Polio 3; Avian infectious bronchitis
28 virus; Human respiratory virus; Transmissible
29 gastro-enteritis virus of swine; Lymphocytic
30

0154902

1 choriomeningitis virus; Lassa virus; Machupo virus; Pichinde
2 virus; Tacaribe virus; Papillomavirus; Simian virus; Sindbis
3 virus, and the like.

4 The lipid vesicle synthetic carriers of the
5 present invention can be used to bind synthetic peptide
6 analogues of bacteria, for example, leprosy, tuberculosis,
7 syphilis and gonorrhea.

8 The lipid vesicle synthetic carriers of the
9 present invention can also be used to bind synthetic peptide
10 analogues of the following parasites: organisms carrying
11 malaria (P. Falciparum, P. Ovace, etc.), Schistosomiasis,
12 Onchocerca Volvulus and other filarial parasites,
13 Trypanosomes, Leishmania, Chagas disease, amoebiasis,
14 hookworm, and the like.

15 The lipid vesicle carriers of the present
16 invention can be used to bind the novel peptides of the
17 present invention corresponding to amino acid sequences in
18 the pre-S region of HBsAg. The lipid vesicle carriers of
19 the present invention can also be used to bind amino acid
20 sequences in the S region, as well as other amino acid
21 sequences for other virus, etc.

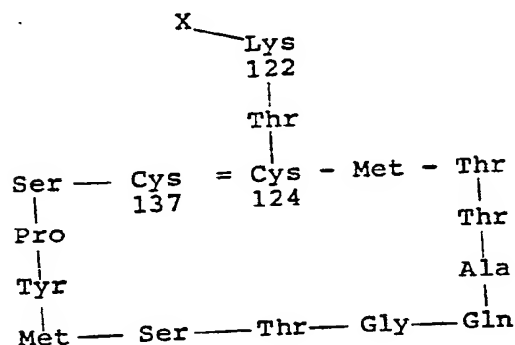
22 Amino acid sequences (corresponding to amino acids
23 in the S region) which contains an antigenic determinant for
24 hepatitis B surface antigen can be linked to the lipid
25 vesicle carrier of the present invention. T.P. Hopp, "A
26 Synthetic Peptide with Hepatitis B Surface Antigen
27 Reactivity", Mol. Imm., 18, 9, 869-872, 1981, propose the
28 following sequence corresponding to the S region of HBsAg:
29 138 139 140 141 142 143 144 145 146 147 148 149
30

0154902

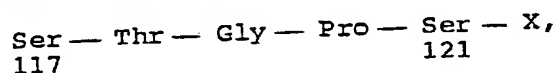
Cys Cys Thr Lys Pro Thr Asp Gly Asn Cys Thr Cys
Other peptides mimicking the antigenic determinant
of HBsAg (S region) include the following:

(1)

Peptide 1



Peptide 2 contains 5 additional amino acid residues:



G.R. Dreesman, Y. Sanchez, I. Ionescu-Matiu, J. T. Sparrow,
H. R. Six, D.L. Peterson, F.B. Hollinger and J.L. Melnick,
"Antibody to Hepatitis B Surface Antigen After A Single
Inoculation of Uncoupled Synthetic HBsAg Peptides", Nature,
295, 158-160, 1982; and (2) the following peptides:

<u>POSITION</u>	<u>SEQUENCE</u>
48-81	Cys-Leu-Gly-Gln-Asn-Ser-Gln-Ser-Pro-Thr-Ser- Asn-His-Ser-Pro-Thr-Ser-Cys-Pro-Pro-Thr-Cys- Pro-Gly-Tyr-Arg-Trp-Met-Cys-Leu-Arg-Arg-Phe- Ile
2-16	Glu-Asn-Ile-Thr-Ser-Gly-Phe-Leu-Gly-Pro-Leu- Leu-Val-Leu-Gln-Cys

0154902

1 22-35 Leu Thr-Arg-Ile-Leu-Thr-Ile-Pro-Gln-Ser-Leu-
2 Asp-Ser-Trp-Cys
3
4 38-52 Ser-Leu-Asn-Phe-Leu-Gly-Gly-Thr-Thr-Val-Cys-
5 Leu-Gly-Gln-Asn
6
7 47-52 Val-Cys-Leu-Gly-Gln-Asn
8
9 95-109 Leu-Val-Leu-Leu-Asp-Tyr-Gln-Gly-Met-Leu-Pro-
10 Val-Cys-Pro-Leu
11
12 104-109 Leu-Pro-Val-Cys-Pro-Leu
13
14 R. Arnon, "Anti-influenza Response Achieved by Immunization
15 With A Synthetic Conjugate", Proc. Natl. Acad. Sci. USA, 79,
16 569-573, 1982. The peptide corresponds to the sequence
17 serine-91 to leucine-108 of the amino acid chain of the
18 virus.
19 A peptide containing an amino acid sequence
20 mimicking the antigenic determinant of polyoma virus medium
21 size tumor antigen is Lys-Arg-Ser-Ars-His-Phe, G. Walter,
22 M.A. Hutchinson, T. Hunter and W. Eckhart, "Purification of
23 Polyoma Virus Medium-Size Tumor Antigen by Immunoaffinity
24 Chromatography", Proc. Natl. Acad. Sci USA, 79, 4025-4029,
25 1982.
26 A peptide containing an amino acid sequence
27 mimicking the antigenic determinant of poliovirus replicase
28 antigen is as follows:
29 Tyr-Ser-Thr-Leu Tyr-Arg-Arg-Trp-Leu-Asp-Ser-Phe
30 450 461,

1 M. H. Baron and D. Baltimore, "Antibodies Against a
2 Synthetic Peptide of the Poliovirus Replicase Protein:
3 Reaction with Native, Virus-Encoded Proteins and Inhibition
4 of Virus-Specific Polymerase Activities In Vitro". Jour.
5 Virology, 43, 3969-3978, 1982.

6 Peptides containing an amino acid sequence
7 mimicking the antigenic determinant of simian virus 40 large
8 tumor antigen are as follows:

9 Met-Asp-Lys-Val-Leu-Asn-Arg and

10 Lys-Pro-Pro-Thr-Pro-Pro-Pro-Glu-Pro-Glu-Thr,

11 G. Walter, K.H. Scheidtmann, A. Carbone, A.P. Laudano and
12 R.A. Lerner, N. Green, H. Alexander, F.-T. Liu, J.G.
13 Sutcliffe and T.M. Shinnick, "Chemically Synthesized
14 Peptides Predicted From the Nucleotide Sequence of the
15 Hepatitis B Virus Genome Elicit Antibodies Reactive With the
16 Native Envelope Protein of Dane Particles", Proc. Natl.
17 Acad. Sci. USA, 78, 6, 3403-3407, 1981.

18 A peptide containing an amino acid sequence
19 mimicking the antigenic determinant of retrovirus R antigen
20 is as follows:

21 Leu-Thr-Gln-Gln-Phe-His-Gln-Leu-Lys-Pro

22 Ile-Glu-Cys-Glu-Pro,

23 J.G. Sutcliffe, T.M. Shinnick, N. Green, F.-T. Liu, H.L.
24 Niman and R.A. Lerner, "Chemical Synthesis of A Polypeptide
25 Predicted From Nucleotide Sequence Allows Detection Of A New
26 Retroviral Gene Product", Nature, 287, 1980.

27 A peptide containing an amino acid sequence
28 mimicking the antigenic determinant of avian sarcoma virus
29 antigen is as follows:
30

0154902

1 Glu-Asp-Asn-Glu-Tyr-Thr-Ala-Arg-Gln-Gly,
2 T.W. Wong and Alan R. Goldberg, "Synthetic Peptide Fragment
3 Of src Gene Product Inhibits the src Protein Kinase and
4 Cross reacts Immunologically With Avian onc Kinases and
5 Cellular Phosphoproteins", Proc. Natl. Acad. USA, 78, 12,
6 7412-7416, 1981.

7 Peptides containing an amino acid sequence
8 mimicking the antigenic determinant of foot-and-mouth
9 disease virus antigen are as follows:

10 141
11 Val Pro Asn Leu Arg Gly Asp Leu Gly Val
12 Leu Ala Gly Lys Val Ala Arg Thr Leu Pro 160
13 and
14 201
15 His Lys Gln Lys Ile Val Ala Pro Val Lys Gln
16 Thr Leu,

17 J.L. Bittle, R.A. Houghten, H. Alexander, T.M. Shinnick,
18 J.G. Sutcliffe, R.A. Lerner, D.J. Rowlands and F. Brown,
19 "Protection Against Foot-And-Mouth Disease By Immunization
20 With A Chemically Synthesized Peptide Predicted From the
21 Viral Nucleotide Sequence", Nature, 298, 30-33, 1982.

22 A peptide containing an amino acid sequence
23 mimicking the antigenic determinant of hemagglutinin X-31
24 (H3N2) influenza virus antigen is as follows:

25 123 125
26 Glu-Gly-Phe-Thr-Trp-Thr-Gly-
27 130 135
28 Val-Thr-Gln-Asn-Gly-Gly-Ser-
29 140
30 Asp Ala-Cys-Lys-Arg-Gly-Pro-
 145 150
 Gly-Ser-Gly-Phe-Phe-Ser-Arg-

151
Leu,

D.C. Jackson, J.M. Murray, D.O. White, C.N. Fagan and G.W. Tregear, "Antigenic Activity of a Synthetic Peptide Comprising the 'Loop' Region of Influenza Virus Hemagglutinin", Virology, 120, 273-276, 1982.

A peptide containing an amino acid sequence mimicking the antigenic determinant of hemagglutinin of type A H3N2 influenza virus antigen was synthesized by G.M. Muller, M. Shapira and R.F. Doolittle, "Antibodies Specific for the Carboxy- And Amino- Terminal Regions of Simian Virus 40 Large Tumor Antigen", Proc. Natl. Acad. Sci USA, 77, 9, 5179-5200, 1980.

A peptide containing an amino acid sequence mimicking the antigenic determinant of influenza virus strain 3QB antigen is Ile₁ Val₁ Asx₂ Thr₁ Ser₂ Glx₂ Pro₁ Gly₃ Ala₁ Leu₁ Lys₁, A. Aitken and C. Hannoun, "Purification of Haemagglutinin and Neuraminidase from Influenza Virus Strain 3QB and Isolation of a Peptide From an Antigenic Region of Haemagglutinin", Eur. J. Biochem, 107, 51-56, 1980.

Peptides containing an amino acid sequence mimicking the antigenic determinant of diphtheria antigen are given as follows:

Natural DT Loop

-Cys-Ala-Gly-Asn-Arg-Val-Arg-Arg-Ser-Val-
186 190 195

Gly-Ser-Ser-Leu-Lys-Cys-
201

Synthetic Peptide

Tetradecapeptide

Gly(188)---Cys-(201)

0154902

1 Hexadecapeptide Cys(186)---Cys-(201)
2 Octadecapeptide Ala-Ala-Cys(186)---Cys-(201)
3 F. Audibert, M. Jolivet, L. Chedid, R. Arnou and M. Sela,
4 "Successful Immunization With a Totally Synthetic Diphtheria
5 Vaccine", Proc. Natl. Acad. Sci. USA, 79, 5042-5046, 1982.

6 A peptide containing an amino acid sequence
7 mimicking the antigenic determinant of Streptococcus
8 pyogenes M antigen is as follows:

9
10 Asn-Phe-Ser-Thr-Ala-Asp-Ser-Ala-Lys
11 10 15
12 Ile-Lys-Thr-Leu-Glu-Ala-Glu-Lys-Ala-Ala-
13 20 25
14 Leu-Ala-Ala-Arg-Lys-Ala-Asp-Leu-Glu-Lys-
15 30 35
16 Ala-Leu-Glu-Gly-Ala-Met

17 E.H. Beachey, J.M. Seyer, D.B. Dale, W.A. Simpson and A.H.
18 Kang, "Type-Specific Protective Immunity Evoked by Synthetic
19 Peptide of Streptococcus Pyogenes M Protein", Nature, 292,
20 457-459, 1981.

21 The lipid vesicle carrier of the present invention
22 can thus be utilized to bind with any amino acid sequence
23 which includes the antigenic determinant for a specific
24 antigen.

25 The lipid vesicle carriers of the present
26 invention can also be used to bind with enzymes.

27 The present invention is also directed to
28 diagnostic tests for direct detection of HBV antigens and
29 HBV antibodies.

30 In order to detect HBV antigens containing
proteins coded for by the pre-S gene in sera of HBV-infected

1 animals such as humans, radioimmunoassay (RIA) or
2 enzyme-linked immunosorbent assay (ELISA) can be employed.

3 One test for detecting HBV antigens according to
4 the present invention is as follows:

5 (1) a solid substrate containing binding sites
6 thereon, e.g., polystyrene beads, is coated with antibodies
7 to a peptide having an amino acid chain corresponding to at
8 least six amino acids within the pre-S gene coded region of
9 the envelope of HBV, the peptide free of an amino acid
10 sequence corresponding to the naturally occurring proteins of
11 HBV;

12 (2) the coated beads are then washed with, for
13 example, tris buffered saline, to remove excess antibody;

14 (3) the beads are then contacted with a protein-
15 containing solution, such as bovine serum albumin (BSA) or
16 gelatin to saturate protein binding sites on the beads (to
17 prevent or reduce non-specific binding) - a convenient
18 concentration of such protein-containing solution can be
19 employed such as 1 mg/ml to 50 mg/ml;

20 (4) beads are then washed to remove excess BSA or
21 gelatin;

22 (5) the beads are then incubated with samples
23 suspected to contain HBV or HBsAg (normal sera is utilized
24 as a control);

25 (6) the beads are then washed with a solution,
26 e.g., tris buffered saline solution, and mixed with a
27 radiolabeled antibody, e.g., I^{125} labeled antibody (antibody
28 to either the peptide or to HBsAg);

29 (7) the beads are then incubated;
30

1 (8) the beads are then washed and counted in a
2 gamma counter.

3 If the specimens have counts at least 2.1 times
4 higher than counts of the control, then the specimens are
5 positive.

6 The pre-S gene coded peptides according to the
7 present invention can be employed as a diagnostic tool to
8 detect antibodies to the pre-S region of HBV in a given
9 sample. The pre-S gene coded peptide, for example, pre-S
10 (120-145), pre-S (12-32), pre-S (32-53), or pre-S (117-134),
11 pre-S(1-21), pre-S(94-117), pre-S(153-171), pre-S(32-53) and
12 pre-S(57-73), is adsorbed on a solid substrate, containing
13 binding sites thereon for example, polystyrene beads. The
14 substrate is thereafter contacted with a substance (protein
15 containing solution), for example, gelatin BSA or powdered
16 milk, to saturate the binding sites thereon. Thereafter,
17 the substrate is washed with a buffered solution and
18 thereafter the buffer is removed. A specimen, e.g., human
19 sera diluted with animal sera is added to the substrate.
20 The resultant mass is then incubated and washed.
21 Thereafter, radiolabeled, e.g., iodinated, e.g., I^{125} ,
22 antibodies to human IgG or IgM is added to the mass. The
23 resultant mass is then washed and counted, e.g., in a
24 gamma-counter. If the count is higher than a count
25 performed on a normal sera control, the specimen contains
26 antibodies to the pre-S region of HBV.
27

28 It is believed that the above procedure for
29 detection of antibodies to the pre-S region of HBV can be
30

1 applied as a diagnostic tool in detecti- hepatitis B virus
2 infection.

3 The pre-S protein moiety appears to be directly
4 involved in attachment of HBV to liver cells of the host.
5 Similar proteins are likely to be involved in the attachment
6 of other viruses, the target of which is the liver. For
7 this reason, synthetic peptides corresponding to the pre-S
8 protein, as well as antibodies to them, could serve as the
9 basis for diagnostic assays of and vaccines against other
10 hepatitis viruses reacting with the same liver receptors as
11 does hepatitis B virus.

12 In the above described procedures involving
13 radioimmunoassay (RIA), an enzyme linked antibody can
14 replace the radiolabeled antibody and ELISA techniques can
15 be performed. Furthermore, fluorescence techniques can be
16 employed in place of RIA or ELISA.

17 The labelling ("marking") of one of the reaction
18 components can be brought about by use of a "marker" or
19 "marker substance" such as by incorporation of a radioactive
20 atom or group, or by coupling this component to an enzyme, a
21 dyestuff, e.g., chromophoric moiety or a fluorescent group.

22 The components concerned are preferably labelled
23 by coupling to an enzyme, since the estimation of this is
24 much simpler than for example, the estimation of
25 radioactivity, for which special apparatus is necessary.

26 The enzymes used are preferably those which can be
27 colorimetrically, spectrophotometrically, or
28 fluorimetrically determined. Non-limiting examples of
29 enzymes for use in the present invention include enzymes
30

0154902

from the group of oxidoreductases, such as catalase,
peroxidase, glucose oxidase, beta-glucuronidase,
beta-D-glucosidase, beta-D-galactosidase, urease and
galactose oxidase.

The coupling of the enzyme and the immunological
component can be brought about in a known way, for example,
by the formation of an amide linkage by methods known from
peptide chemistry.

The labelling with a radioactive isotope can also
be performed in a known way. Isotopes useful for labelling
are predominantly I^{125} , I^{131} , C^{14} , and H^3 .

The incubation steps utilized in carrying out the
above procedures can be effected in a known manner, such as
by incubating at temperatures of between about 20°C and
about 50°C for between about 1 hour and about 48 hours.

Washings as described above are typically effected
using an aqueous solution such as one buffered at a pH of
6-8, preferably at a pH of about 7, employing an isotonic
saline solution.

The present invention also concerns diagnostic
test kits for conducting the above-described methods for
detecting antigens and antibodies.

A diagnostic test kit according to the present
invention for detecting antigens coded for the pre-S gene of
HBV in a test sample, would include the following:

a. a solid substrate coated with antibodies to a
peptide having an amino acid chain corresponding to at least
six consecutive amino acids within the pre-S gene coded
region of the envelope of HBV, the peptide free of an amino

1 acid sequence corresponding to the naturally occurring
2 proteins of HBV,

3 b. a protein-containing solution to saturate
4 protein binding sites on the solid substrate, and

5 c. a given amount of radiolabeled antibody, such
6 antibody to either the peptide or HBsAg.

7 A diagnostic test kit according to the present
8 invention for detecting antibodies to the pre-S region of
9 hepatitis B virus in a test sample, would include the
10 following:

11 a. a solid substrate having adsorbed thereon a
12 peptide having an amino acid chain corresponding to at least
13 six consecutive amino acids within the pre-S gene coded
14 region of the envelope of HBV, the peptide free of an amino
15 acid sequence corresponding to the naturally occurring
16 proteins of HBV, the substrate being exposed to a
17 protein-containing solution to saturate protein binding
18 sites on the solid substrate, and

19 b. a given amount of radiolabeled antibodies to
20 human IgG or IgM.

21 Radiolabeled antibodies used in the
22 above-described test kits can be packaged in either solution
23 form, or in lyophilized forms suitable for reconstitution.

24 In the above test kits, enzyme or fluorescent
25 labelled antibodies can be substituted for the described
26 radiolabeled antibodies.

27 The above described process and test kit for
28 detection of antibodies to the pre-S region of hepatitis B
29 virus can be utilized in many applications, such as
30

1 (1) detecting HBV infection in a patient by
2 taking serum from the patient and applying the above
3 described test or using the above described test kit; and

4 (2) predicting recovery from HBV infection by
5 taking serum from an infected patient and applying the above
6 described antibody detection procedures.

7 The above described test procedure and test kit
8 for antibody detection can be used for making qualitative
9 comparisons between different HBV vaccines by taking serum
10 from vaccinated patients and then utilize the
11 above-described test procedure or kit for antibody
12 detection. In general all known immunoassays using this
13 antigen as reagent can be performed using the synthetic
14 peptide of this invention. Generally all known immunoassays
15 using antibody containing serum or reagents can be performed
16 using antibody serum produced through the use of a synthetic
17 peptide of this invention. These immunoassays included all
18 those disclosed by Langone and Van Vunakis, Methods of
19 Enzymology, Academic Press, Volumes 70, 73 and 74. Those
20 assays disclosed in the disclosures of the following U.S.
21 Patents: 4,459,359; 4,343,896; 4,331,761; 4,292,403;
22 4,228,240; 4,157,280; 4,152,411; 4,169,012; 4,016,043;
23 3,839,153; 3,654,090 and Re 31,006 and volumes 70, 73 and 74
24 of Methods of Enzymology are incorporated herein by
25 reference.
26

27 A hepatitis B vaccine can be prepared by directly
28 using a conjugate of a lipid vesicle and a peptide
29 containing an amino acid chain corresponding to at least six
30 consecutive amino acids within the pre-S gene coded region

0154902

1 of the surface antigen of hepatitis B virus in an
2 appropriate buffer. The conjugate having peptide in the
3 appropriate concentration can be used as a vaccine with or
4 without an adjuvant, such as, e.g., aluminum hydroxide or
5 others.

6 The active component of the vaccine can be
7 employed with a physiologically acceptable diluent (medium),
8 e.g., phosphate buffered saline. Generally speaking, the
9 synthetic peptide concentration in a physiologically
10 acceptable medium will be between approximately less than 1
11 miligram and more than 10 micrograms per dose.

12 The vaccine can be prepared and used in the same
13 general manner as disclosed in U.S.P. 4,118,479, the entire
14 contents of which are incorporated by reference herein.

15 The vaccine can be administered by subcutaneous,
16 intradermal or intramuscular injection. While the preferred
17 route would depend upon the particular vaccine, it is
18 believed that intramuscular injection will be generally
19 suitable. Frequency of administration will vary depending
20 upon the vaccine. Generally speaking, the vaccine will be
21 administered in two doses about one month apart followed by
22 a booster at six months to one year after primary
23 immunization. The subsequent doses or the booster will
24 depend on the level of antibody in the blood as a result of
25 the initial immunization, and in certain instances may be
26 unnecessary.

27 The hepatitis vaccine of the present invention is
28 recommended for all persons at risk of developing hepatitis
29 B infection and particularly those at especially high risk
30

0154902

1 such as patients and staff on hemodialysis unit, medical
2 , personnel, persons of tropical populations and those
3 visiting the tropics. In the case of tropical populations,
4 particularly in Africa, Asia, the Mediterranean region and
5 South America, where high incidence of hepatitis B
6 infections has been consistently observed, the vaccine
7 should be administered sufficiently early in life to prevent
8 acquisition of chronic carrier state infection which tend to
9 occur in these regions within the first five years of life.
10 In fact, the vaccine is expected to be useful for all
11 persons not already protected against hepatitis B infections
12 as a result of prior immunity.
13

14 In order to more fully illustrate the nature of
15 the invention and the manner of practicing the same, the
16 following non-limiting examples are presented:

17 EXAMPLES

18 Example 1

19 SDS-Polyacrylamide Gel Electrophoresis Of HBsAg.

20 About 20 and 200 ug, respectively, of HBsAg were
21 separately electrophoresed for silver staining and transfer
22 to nitrocellulose, respectively. Before electrophoresis,
23 HBsAg was treated for 30 minutes at 37°C with
24 2-mercaptoethanol and NaDodSO₄ (10 mg/ml each in 8 M urea,
25 0.0625 M Tris, pH 7.2). Similar results were obtained with
26 HBsAg alkylated with iodoacetate after reduction. HBsAg was
27 purified and radiolabeled as described (A.R. Neurath, N.
28 Strick, C.Y. Huang, Intervirology, 10, 265 (1978)).

29 SDS-Polyacrylamide gel electrophoresis
30 ("SDS-PAGE") was carried out following published procedures.

1 See V.K. Laemmli, Nature (London), 227, 680 (1970).

2 However, in order to maintain proteins in fully denaturated
3 form, 8M urea was utilized in the running buffers in
4 electrophoresis.

5 Polypeptides separated by SDS-PAGE were
6 transferred to nitrocellulose using the TE 42 Transphor unit
7 9 (Hoefer Scientific Instruments, San Francisco, California)
8 following the procedure recommended by the manufacturer. The
9 transferred proteins were tested for determinants reacting
10 with antibodies to intact HBsAg (anti-HBs) using
11 ^{125}I -labeled human anti-HBs supplied as part of a commercial
12 test kit (Abbott Laboratories, North Chicago, Illinois) as
13 described (J.C. McMichael, L.M. Greisiger, L. Millman, J.
14 Immunol. Meth., 45, 79, (1981)).

15 From the 20ug sample gel, separated HBsAg
16 polypeptides (their M_r given in kilodaltons) were stained by
17 silver in situ (J.H. Morrissey, Anal. Biochem, 117, 307,
18 (1981)), (see Fig. 1, Panel a) to yield two major and
19 several minor polypeptides as expected. The separated
20 polypeptides from the other 200 μg sample gel was then
21 electrophoretically transferred to nitrocellulose, reacted
22 (probed) with ^{125}I -labeled antibodies to intact HBsAg (anti
23 HBs) and submitted to autoradiography (Fig. 1b).

24 Surprisingly, the 33 and 36 kilodalton (P33 and
25 P36), rather than the two most abundant polypeptides reacted
26 preferentially with anti-HBs (Fig. 1, Panel b). This
27 suggested the presence of disulfide bond independent
28 antigenic determinants reacting with anti-HBs on amino acid
29 sequences which are not coded for by the S-gene of HBV DNA.
30

1 P33 and P36 contain the sequence corresponding to the
2 product of the S-gene and additional 55 residues at the
3 amino-terminal part starting with Met at position 120 in the
4 pre-S gene region (See Fig. 2).

5 Example 2

6 Synthesis Of A Peptide Mimicking Antigenic
7 Determinants Corresponding To Residues 120-145 Of The Pre-S
8 Gene Product

9 The location of antigenic determinants on proteins
10 may be predicted from computing the relative hydrophilicity
11 along the amino acid sequence. See T.P. Hopp, K.R. Woods,
12 Proc. Natl. Acad. Sci. USA, 78, 3824 (1981) and J. Kyte,
13 R.F. Doolittle, J. Mol. Biol., 157, 105 (1982).

14 Results of such computation (J. Kyte et al supra) for the
15 translation product of the pre-S region are shown in Fig. 3
16 and suggest the location of antigenic determinants in the
17 sequence to the right from Met 120 within residues 120-140.
18 The segment corresponding to residues 120-145 (Fig. 2)
19 (pre-S 120-145, subtype adw₂) was selected for synthesis.

20 A C-terminal Cys(-SH containing) residue was added
21 to allow unambiguous conjugation to carrier molecules and
22 affinity matrices, while leaving the N-terminal unblocked as
23 it may be in the intact protein. The molecule contains one
24 Tyr and can therefore be radiolabeled. The Tyr could also be
25 used for conjugation, although it might be a part of the
26 antigenic determinant.
27

28 The peptide was synthesized by an accelerated
29 version of stepwise solid phase peptide synthesis on the
30 benzhydrylamine-type resin of Gaehde and Matsueda (Int. J.

0154902

1 Peptide Protein Res., 18, 451, (1981)) using
2 Boc-NH-CH(phenyl)-phenyl-OCH₂COOH to derivatize NH₂CH₂-Resin
3 (A.R. Mitchell, S.B.H. Kent, M. Engelhard and R.B.
4 Merrifield, J. Org. Chem., 43, 2845-2852, (1978)). After the
5 Cys was coupled, the protected peptide chain was assembled
6 according to the following protocol:

7 1. Deprotection: 65% v/v trifluoroacetic acid in
8 dichloromethane, 1x10 minutes;

9 2. Wash: a flowing stream of dichloromethane was
10 run over the resin under suction from an aspirator for 20
11 seconds;

12 3. Neutralization: 10% v/v diisopropylethylamine
13 in dichloromethane, 2x1 minutes;

14 4. Wash: a flowing stream of dichloromethane was
15 run over the resin under suction from an aspirator for 20
16 seconds;

17 5. Coupling: 2 mmol tert.Boc-L-amino acid in 2ml
18 dichloromethane was added to the neutralized resin followed
19 immediately by 1mmol dicyclohexylcarbodiimide in 2ml
20 dichloromethane; after 10 minutes a sample of resin
21 (approximately 5mg) was taken for determination of coupling
22 yield by quantitative ninhydrin, and 10ml dimethylformamide
23 was added and the coupling continued. (Asn and Gln were
24 coupled in the presence of hydroxybenzotriazole).

25 6. After the ninhydrin determination of a
26 satisfactory coupling, the resin was washed as in step 4,
27 above. For the addition of subsequent residues, the cycle
28 was repeated. If recoupling was necessary, steps 3-5 were
29 repeated. The synthesis was performed on a 0.5mmol scale
30

0154902

1 0.5gram aminomethyl-resin of 1mmol/g loading). All volumes
2 were 10ml except where noted.

3 Protected amino acid derivatives used were
4 N-alpha-tert.butylloxycarbonyl protected and side chain
5 protected as follows: Arg(N^GTosyl); Cys(4MeBzl); Tyr(BrZ);
6 Asp(OBzl); Thr(Bzl); His(ImTosyl). Met and Trp were
7 unprotected on the side chains. In another synthesis,
8 otherwise identical, use of His(ImDNP) and Trp(InFormyl)
9 gave purer product.

10 Assembly of the peptide chain was monitored by the
11 quantitative ninhydrin reaction (V.K. Sarin, S.B.H. Kent,
12 J.P.Tam, R.B. Merrifield, Anal. Biochem, 117, 147-157,
13 (1981)) and was without difficulty except for the addition
14 of the histidine residue which was 10% incomplete despite
15 repeated couplings, presumably due to an impure amino acid
16 derivative. After assembly of the protected peptide chain,
17 the N-terminal Boc group was removed by trifluoroacetic acid
18 treatment and the resin neutralized as in steps 1-4 above.
19 Then the peptide was cleaved and deprotected by a 1 hour
20 treatment at 0°C with HF containing 5% v/v p-cresol and 5%
21 v/v p-thiocresol to give the desired peptide as the
22 C-terminal cysteinamide. Where His(ImDNP) was used, the DNP
23 was removed by treatment with phenylphenol prior to HF
24 cleavage. Where Trp (InFormyl) was used, HF conditions were
25 adjusted to remove the Formyl group; either HF containing
26 10% anisole and 5% 1,4-butanedithiol, or HF containing
27 p-cresol and 5% 1,4-butanedithiol. The product was
28 precipitated and washed by the addition of ether, then
29 dissolved in 5% v/v acetic acid in water and lyophilized to
30

1 give a fluffy white solid

2 Quantitative Edman degradation (H.D. Niall, G.W.
3 Tregear, J. Jacobs, Chemistry and Biology of Peptides, J.
4 Meienhofer, Ed (Ann Arbor Press, Ann Arbor, MI, 1972), pp.
5 659-699) of the assembled peptide-resin revealed a high
6 efficiency of chain assembly (S.B.H. Kent, M. Riemen, M.
7 LeDoux, R.B. Merrifield, Proceedings of the Fourth
8 International Symposium on Methods in Protein Sequence
9 Analysis, M. Elzinga, Ed. (Humana, Clifton, New Jersey,
10 1982), pp. 626-628) which proceeded at a ≥ 9.7 percent
11 efficiency at each step, except for histidine at sequence
12 position pre-S 128. HPLC of the peptide cleaved off the
13 resin revealed a single major peak corresponding to
14 approximately 85 percent of peptide material absorbing light
15 at 225 nm.

16 Examples 3-6

17 Immunologic Properties Of A Peptide Mimicking
18 Antigenic Determinants Corresponding To Residues 120-145 of
19 the Pre-S Gene Product (pre-S 120-145)
20

21 Example 3

22 Immunization

23 Immunization of rabbits with either free or
24 carrier-bound pre-S 120-145 (subtype adw₂) were conducted
25 and resulted in an antibody response in all animals against
26 both the homologous peptide and HBsAg (Fig. 4).

27 The peptide corresponding to the amino acid
28 sequence 120-145 (pre-S 120-145) of the pre-S region of HBV
29 DNA (subtype adw₂; P. Valenzuela, P. Gray, M. Quiroga, J.
30

1 Zaldivar. H.M. Goodman, W.J. Rutter, Nature (London), 280,
2 815, (1979)) containing an additional Cys residue at the
3 C-terminal, added for convenience of coupling to carriers,
4 was synthesized by an improved solid phase technique (S.B.H.
5 Kent, Biomedical Polymers, E.P. Goldberg, A. Nakajima, Eds.
6 (Academic, New York, 1980), pp. 213-242; A.R. Mitchell,
7 S.B.H. Kent, M. Engelhard, R.B. Merrifield, J. Org. Chem.
8 43, 2845, (1978); and S. Mojsov, A.R. Mitchell, R.B.
9 Merrifield, J. Org. Chem., 45, 555 (1980).

10 For immunoassays and linking to carriers the
11 peptide was treated with 2-mercaptoethanol and separated
12 from low M_r components by chromatography on Sephadex G-10
13 (A.R. Neurath, S.B.H. Kent, N. Strick, Proc. Natl. Acad.
14 Sci. USA, 79, 7871 (1982)).

15 Groups of two to three rabbits were immunized with
16 either free pre-S 120-145 or with the peptide linked to
17 cysteine-activated liposomes containing stearylamine,
18 dilauroyl lecithin and cholesterol which had been fixed with
19 glutaraldehyde, and either did or did not have attached RAT
20 groups for enhancing antibody responses to haptens (A.R.
21 Neurath, S.B.H. Kent, N. Strick, J. Gen. Virol., in press
22 (1984)). The immunization schedule involving the use of
23 complete and incomplete Freund's adjuvant was the same as
24 described (Neurath, Kent, Strick, et al (1984) supra).
25 Antibodies to HBsAg in sera of rabbits immunized with pre-S
26 120-145 were tested by a double-antibody radioimmunoassay
27 (RIA) using HBsAg-coated polystyrene beads and ^{125}I -labeled
28 anti-rabbit IgG (Neurath, Kent, Strick, et al (1984) supra).
29
30

0154902 .

1 Antibodies to the homologous peptide were tested ~
2 by a similar test except that 2.5 mg of a cellulose-peptide
3 conjugate were used instead of coated beads. This conjugate
4 was prepared in the following way: 0.5 g of sulfhydryl
5 cellulose, prepared as described (P.L. Feist, K.J. Danna,
6 Biochemistry, 20, 4243 (1981)), were suspended in 5 ml 0.1 M
7 sodium acetate, pH 5, and mixed with 2.5 ml of 0.25 M
8 N-N'-p-phenylenedimaleimide in dimethylformamide for one
9 hour at 30°C and then washed with 0.1 M phosphate-10mM EDTA,
10 pH 7.0. The cellulose derivative was suspended in 10 ml of
11 the latter buffer containing 5 mg of pre-S 120-145 and mixed
12 for at least sixteen hours at 20°C. The cellulose derivative
13 was extensively washed and suspended in 0.14 M NaCl-10 mM
14 Tris-3 mM NaN₃ (TS). The final preparation contained 8 mg of
15 pre-S 120-145 per g of cellulose.
16

17 Example 4

18 Radioimmunoassays were conducted with several
19 dilutions of a serum from one of the rabbits immunized with
20 pre-S 120-145 linked to liposomes (See Fig. 5).
21

22 Antibodies were still detectable when the antisera
23 were diluted up to 1.6×10^5 -fold (Fig. 5).

24 Pre-S 120-145 or anti-pre-S 120-145 inhibited the
25 reaction between ¹²⁵I-labeled anti-HBs and P33 (P36).
26 ¹²⁵I-labeled HBsAg was immunoprecipitated with anti-pre-S
27 120-145 at all dilutions positive by RIA (Fig. 5). HBV
28 particles reacted with anti-pre-S 120-145 as determined by
29 detection of HBV-DNA within the immune complexes and by
30

1 electron microscopy (A.R. Neurath, N. Strick, L. Baker, S.
2 Krugman, Proc. Nat. Acad. Sci. USA, 79, 4415 (1982)).
3

4 Example 5

5 Anti-Peptide Antibody as A Specific Probe for 6 Detection of P33 and P36

7 Anti-pre-S 120-145 was reacted with P33 and P36.
8 HBsAg polypeptides separated by SDS-PAGE run in urea were
9 transferred to nitrocellulose, reacted with anti-pre-S
10 120-145 diluted 1/80 in TS containing 10 mg/ml of bovine
11 serum albumin and 2.5 mg/ml of gelatine (TS-BG) for five
12 hours at 20°C. To detect bound IgG, the nitrocellulose sheet
13 was washed and exposed to ¹²⁵I-labeled protein A (0.4 µC/100
14 ml TS-BG) for five hours at 20°C. For further details see
15 Fig. 1. In Fig. 6, arrows indicate the positions of P33 and
16 P36. The top arrow (corresponding to a molecular weight of
17 66 kilodaltons) indicates another protein reacting with
18 anti-pre-S 120-145, possibly corresponding to a dimer of
19 P33.
20

21 Example 6

22 Development Of A Diagnostic Test For The 23 Detection Of Antigens Coded For By The Pre-S Gene In Sera 24 Of HBV-Infected Individuals

25 Fig. 7 shows the results of a diagnostic test
26 based on polystyrene beads coated with anti-pre-S 120-145.
27

28 Serial dilutions of an HBsAg-positive serum in a
29 mixture of normal human and rabbit serum each diluted 1/10
30 in TS were tested. ¹²⁵I-labeled human anti-HBs (Abbott

1 Laboratories) was used in the test performed essentially as
 2 described for the AUSRIA II diagnostic kit (Abbott
 3 Laboratories). Results are expressed as RIA ratio units,
 4 determined by dividing cpm corresponding to positive samples
 5 by cpm corresponding to positive samples by cpm
 6 corresponding to normal serum controls. The endpoint titer
 7 corresponds to the highest dilution at which the RIA ratio
 8 was 2.1 (broken line). The endpoint titer of the serum as
 9 determined by the AUSRIA test was approximately $1/10^6$.
 10 Negative results were obtained with control beads coated
 11 with normal rabbit IgG.

12 Similar results were obtained with sera containing
 13 HBsAg subtypes ad and ay, indicating that the synthetic
 14 peptide with the sequence corresponding to subtype adw (Fig.
 15 2) carried common group-specific antigenic determinants.

17 Example 7

18 Synthesizing and Testing 19 S(135-155) Derivatives

20 Each of the conjugates ((1) to (26)) of S(135-155)
 21 listed in Table 1, except conjugate 3, was mixed 1:1 with
 22 complete Freund's adjuvant and injected into two New Zealand
 23 White rabbits (65 to 160 µg of peptide per rabbit). The
 24 rabbits were further injected at biweekly intervals with
 25 equal doses of conjugates in incomplete Freund's adjuvant
 26 (not used for conjugate 3). Blood specimens were taken two
 27 weeks after each injection.

28 To prepare conjugates 1, and 4-8 (Table 1), 1 mg
 29 quantities of peptide 309-329 of the env gene product
 30 (S(135-155)) were activated with a two times molar excess o

0154902

1 N-ethyl-N'(dimethyl-aminopropyl) carbodiimide (EDAC) and
2 N-hydroxy-benzotriazole (NHBT) and subsequently linked to
3 equimolar quantities of poly-D-lysine and diaminoalkanes
4 (from Fluka AG, Buchs, Switzerland), respectively, as
5 described (Arnon, R., Sela, M., Parant, M. and Chedid, L.,
6 "Antiviral Response Elicited By A Completely Synthetic
7 Antigen With Built-In Adjuvanticity", Proceedings of The
8 National Academy of Science USA, 77,6769-6772, (1980)). To
9 prepare conjugates 2 and 3, 1 mg quantities of each
10 EDAC-activated S(135-155) and MDP (Calbiochem, San Diego,
11 California) were linked to 10 mg of poly-D-lysine. Peptide
12 309-329 of the env gene product (800µg) was oxidized with
13 ferricyanide (Dreesman et al, 1982 supra), activated with
14 EDAC as above and linked to 4 mg of LPH. Chromatography on
15 Sephadex G-25 indicated complete linking of the peptide to
16 LPH (conjugate 9). The oxidized, EDAC-activated peptide (1
17 mg) was also conjugated to 1 mg of polyvaline in a
18 suspension of 2.5 ml of 1 M NaHCO₃, pH 8.5, and 10 ml of
19 CHCl₃. The interphase and aqueous phase after centrifugation
20 was used for immunization (conjugate 10).
21

22 Liposomes were prepared by the method of Oku, N.
23 Scheerer, J.F., and MacDonald, R.C., "Preparation of Giant
24 Liposomes", Biochimica et Biophysica Acta, 692, 384-388
25 (1982). Stearylamine, dilauroyl lecithin and cholesterol
26 were dissolved in glucose-saturated ethanol at final
27 concentrations of 10, 23 and 1.43 mg/ml, respectively. For
28 some liposome preparations, the concentration of dilauroyl-
29 lecithin was decreased to 17.5 mg/ml and sphingomyelin was
30 added (10 mg/ml). Other preparations contained as an

0154902 .

1 additional component lipid A (420µg/ml; Calbiochem). The
2 solutions were dialyzed against 0.1 M NaCH₃O₃, pH 8.5, in
3 dialysis bags with a molecular weight cut-off of 10³ daltons
4 for at least sixteen hours. The liposomes were treated for
5 approximately six hours with glutaraldehyde (final
6 concentration 30 mg/ml), mixed with 0.5 volumes of 33.9%
7 (w/w) sodium diatrizoate, floated four times into 1 M NaCH₃O₃
8 by centrifugation for ten minutes at 10,000 rpm, and reacted
9 with 0.84 to 1 mg of peptide 309-329 of the env gene product
10 per 10 mg stearylamine overnight at 20°C. The linking of
11 peptide 309-329 of the env gene product to liposomes under
12 these conditions was complete. Some preparations were
13 reacted additionally with 7.5 mg of RAT (Biosearch, San
14 Rafael, California) per 10 mg of stearylamine for six hours
15 at 20°C. The liposomes were floated three times into 0.14 M
16 NaCl, 0.01 Tris-HCl-0.02% NaN₃ (TS) and dialyzed against
17 TS-10⁻⁴ M oxidized glutathione for at least sixteen hours.
18

19 In some cases (20) and (21) the stearylamine-
20 containing liposomes were not derivatized with GA but
21 instead directly reacted with EDAC-activated peptide 309-329
22 of env gene product. Alternately, (18) and (19), the
23 activated peptide 309-329 of env gene product was linked to
24 glutaraldehyde-treated liposomes further derivatized by
25 reaction with 0.2 M ethylene diamine at pH 8.5 overnight at
26 20°C followed by floating two times into 0.1 M NaHCO₃, pH
27 8.5, reduction with 10µM sodium dithionite for one hour at
28 20°C and repeated floating into the same buffer. An aliquot
29 of these liposomes was additionally reacted with
30

1 EDAC-activated RAT. The liposomes were finally dialyzed
2 against $TS-10^{-4}$ M oxidized glutathione.

3 In one preparation (22), stearic acid was used
4 instead of stearylamine for the preparation of liposomes.
5 These were dialyzed against 0.01 M NaCl, activated with EDAC
6 (50 mg/ml for two hours plus additional 25 mg/ml for one
7 hour) at pH 5.5 and 20°C, floated two times into 0.01 M NaCl
8 and reacted with the peptide 308-329 of the env gene product
9 in 1 M $NaHCO_3$, pH 8.5, overnight.

10 Polyglutaraldehyde microspheres were prepared as
11 described by Margel, S., Zisblatt, S. and Rembaum, A.
12 "Polyglutaraldehyde: A New Reagent For Coupling Proteins To
13 Microspheres And For Labeling Cell-Surface Receptors. II.
14 Simplified Labeling Method By Means Of Non-Magnetic And
15 Magnetic Polyglutaraldehyde Microspheres", Journal of
16 Immunological Methods, 28, 341-353 (1979), using Polysurf
17 10-36 B (Bartig Industries Inc., New Canaan, Conn., Margel &
18 Offarim, (1983)). One mg of the peptide 309-329 of the env
19 gene product was linked to approximately 50 mg of
20 microspheres under conditions similar to those described for
21 glutaraldehyde treated liposomes. Conjugate 25 was prepared
22 by treating the microspheres with 5 ml of 0.1 M ϵ -amino
23 caproic acid at pH 8.5 overnight. After centrifugation, the
24 microspheres were suspended in dimethylformamide (2ml) and
25 reacted with 2 mg EDAC plus 670 ug NHBTA for one hour at
26 20°C. After centrifugation, the microspheres were
27 resuspended in 2 ml of 0.1 M $NaHCO_3$, pH 8.5, containing 1 mg
28 of peptide 309-329 of the env gene product.
29
30

0154902

1 All reagents listed above were of analytical grade
2 and obtained from Sigma, St. Louis, Missouri, unless
3 indicated otherwise.

4 Free peptide 309-329 of the env gene product (mol.
5 weight = 2,664 daltons) containing five cysteine residues
6 was in a predominantly monomeric form, since it was eluted
7 after molecular exclusion chromatography in about the same
8 fractions as insulin A chain. Linking to diaminobutane and
9 to other diamino-alkanes (data not shown) resulted in
10 formation of S(135-155) polymers which were immunogenic and
11 induced both antipeptide and anti-HBs antibodies.
12 Preparations (4), (5) and (7) also induced anti-HBs, while
13 polymers with diaminooctane or dodecane linkers (6) and (8)
14 failed to do so (Fig. 8) for reasons not known. Oxidation of
15 the peptide 309-329 of the env gene product resulted in
16 polymerization (data not shown). The polymer linked to LPH
17 (conjugate 9) induced high levels of anti-S(135-155) but no
18 anti-HBs, unlike S(135-155) linked to KLH or LPH in its
19 reduced form (Neurath et al., 1982, supra). This finding
20 again emphasizes the role of peptide conformation in
21 inducing antibodies to the native protein. Linking of the
22 oxidized peptide to highly hydrophobic poly-L-valine
23 resulted in a conjugate (10) of low immunogenicity.
24 S(135-155) linked to poly-D-lysine administered with
25 Freund's adjuvant (1) or having covalently linked MDP and
26 given without adjuvant (3) induced both anti-S(135-155) and
27 anti-HBs. The latter conjugate administered with Freund's
28 adjuvant (2) appeared poorly immunogenic. S(135-155) linked
29 to glutaraldehyde treated liposomes containing stearylamine
30

1 (conjugate 11) induced levels of anti-HBs comparable to
2 those elicited by those elicited by conjugates with KLH or
3 LPH (Neurath et al., 1982, supra). Incorporation of
4 sphingomyelin and/or lipid A, components reported to enhance
5 the antigenicity of haptens inserted into liposomal
6 membranes (Yasuda, T., Dancey, G.F. and Kinsky, S.C.,
7 "Immunogenicity Of Liposomal Model Membranes In Mice:
8 Dependence On Phospholipid Composition", Proceedings Of The
9 National Academy Of Sciences, 74, 1234-1236 (1977)), into
10 the liposomes (conjugates 13, 15a, 16) failed to enhance
11 anti-HBs, responses.

12 Conjugates (18 and 19) prepared by linking
13 S(135-155) to glutaraldehyde-treated liposomes through an
14 ethylenediamine bridge rather than directly, had the
15 capacity to induce anti-HBs but a considerable variability
16 in response between individual rabbits was observed.

17 S(135-155) before or after oxidation and
18 subsequently linked to stearyl-amine-containing liposomes
19 (not fixed with glutaraldehyde; preparations 20 and 21) or
20 to stearic acid-containing liposomes (22) induced low levels
21 of anti-S-135-155 and no measurable anti-HBs.
22

23 S(135-155) linked directly to microspheres of
24 polyglutaraldehyde (preparations 23 and 24) induced a
25 primary anti-HBs response. However, the level of anti-HBs
26 decreased in the course of immunization. Anti-HBs was un-
27 detectable in sera collected two weeks after the third
28 immunization. S(135-155) linked to these microspheres
29 through ϵ -amino-caproic acid (25) and l-cysteine (26)
30

1 bridges, respectively, either failed (25) or was marginally
2 efficient (26) in eliciting anti-HBs.

3 S(135-155)-KLH or LPH conjugates elicited a
4 primary anti-HBs response but the level of anti-HBs failed
5 to increase in sera of rabbits after additional antigen
6 doses (Neurath et al., 1982 supra). With the conjugates
7 described above, generally, a decrease of anti-HBs levels
8 was observed four or six weeks after primary immunization
9 (Fig. 9B), but exceptions were observed in a minority of
10 rabbits (panel 5, Fig. 9A). This declining trend was
11 uniformly reversed when RAT was inserted into liposomal
12 membranes together with S(135-155) (for example Fig. 9C and
13 Fig. 9D).

14 The immunogenicity of haptens inserted into
15 liposomal membranes depends on the phospholipid composition
16 of the liposomes and seemed to be inversely related to the
17 fluidity of these membranes (Yasuda et al., 1977 supra;
18 Dancey, G.F., Yasuda, T. and Kinsky, S.C. , "Effect Of
19 Liposomal Model Membrane Composition On Immunogenicity", The
20 Journal Of Immunology, 120, 1109-1113 (1978)).

21 Treatment of stearylamine-containing liposomes
22 with glutaraldehyde was found to provide reactive groups
23 suitable for linking of synthetic peptides and at the same
24 time increases the rigidity of the lipid membranes. Such
25 liposomes, especially when containing carrier function
26 enhancing RAT sites (Alkan, S.S., Nitecki, D.E. and Goodman,
27 J.W., "Antigen Recognition And the Immune Response: The
28 Capacity of 1-Tryosine-Azobenzenearsonate To Serve As A
29 Carrier For A Macromolecular Hapten", The Journal Of
30

1 Immunology, 107, 353-358, (1971), and Alkan, S.S., Williams,
 2 E.B., Nitecki D.E. and Goodman, J.W.. "Antigen Recognition
 3 And the Immune Response. Humoral And Cellular Immune
 4 Responses To Small Mono- And Bifunctional Antigen
 5 Molecules", The Journal Of Experimental Medicine, 135,
 6 1228-1246, (1972)), are a promising tool for preparing fully
 7 synthetic immunogens for eliciting anti-viral antibodies.

9 TABLE 1

10 List of cross-linkers and carriers used
 11 for the preparation of S(135-155) conjugates

- 12
- | | | |
|----|-------|--|
| 13 | (1) | Poly-D-lysine (mol. weight $3-7 \times 10^4$) |
| 14 | (2) | 1 + N-Acetylmuramyl-L-alanyl-D-isoglutamine (MDP) |
| 15 | (3) | = 2 |
| 16 | (4) | 1,4-diaminobutane |
| 17 | (5) | 1,6-diaminohexane |
| 18 | (6) | 1,8-diaminooctane |
| 19 | (7) | 1,10-diaminodecane |
| 20 | (8) | 1,12-diaminododecane |
| 21 | (9) | Oxidized S(135-155) linked to LPH |
| 22 | (10) | Oxidized S(135-155) linked to poly-L-valine |
| 23 | (11) | Liposomes containing stearylamine, and treated
24 with glutaraldehyde |
| 25 | (12) | = 11 = L-tyrosine-azobenzene-p-arsonate (RAT) |
| 26 | (13) | = 11 + Sphingomyelin (from bovine brain) |
| 27 | (14) | = 13 + RAT |
| 28 | (15a) | = 11 + Lipid A |
| 29 | (15) | = 15a + RAT |

30

0154902

- 1 (16) = 13 + Lipid A
- 2 (17) = 16 + RAT
- 3 (18) = 11 treated with ethylenediamine
- 4 (19) = 18 + RAT
- 5 (20) = Liposomes containing stearylamine reacted
- 6 with oxidized S(135-155) (see 9)
- 7 (21) = 20 except S(135-155) was oxidized after
- attachment to liposomes
- 8 (22) Stearic acid containing liposomes
- 9 (23) Polyglutaraldehyde micropheres
- 10 (24) = 23 + RAT
- 11 (25) = 23 treated with ϵ -aminocaproic acid
- 12 (26) = 23 treated with L-cysteine

14 Example 8

15 A peptide pre-S (12-32) (subtype adw₂) was
16 synthesized according to the procedure described hereinabove
17 in Example 2. The free peptide, the peptide linked to
18 glutaraldehyde cross-linked liposomes (\pm RAT groups)
19 (according to the procedure described above in Example 7) as
20 well as the peptide linked to KLH were used to immunize
21 rabbits. The corresponding antibodies recognized not only
22 the peptide, but also HBsAg and HBV. In view of the above,
23 this peptide is believed quite useful for a vaccine against
24 hepatitis B virus, and as the basis of useful HBV
25 diagnostics based on either the peptide itself (to detect
26 anti-HBV response in infected or immunized individuals), or
27 on peptide antibodies to detect hepatitis B antigens.

1 Example 9

2 A peptide pre-S (117-134) (subtype adw₂) was
3 synthesized according to the procedure described hereinabove
4 in Example 2.

6 Example 10

7 A rabbit was immunized with the peptide pre-S
8 (117-134) prepared according to Example 9 and linked to a
9 carrier according to the procedure of Example 7. Such
10 immunization was conducted according to the procedure
11 described hereinabove in Example 3 and was found to produce
12 antibodies in the serum of the rabbit so innoculated.
13 However, the antibody titers were substantially less than
14 those observed for the use of pre-S (120-145) and pre-S
15 (12-32).

17 Example 11

18 The immune response in rabbits to each of two
19 synthetic peptides corresponding to residues 120-145 and
20 12-32 of the translational product of the pre-S gene of HBV
21 DNA (subtype adw₂) was tested. Peptide pre-S (120-145) was
22 prepared according to Example 2 and peptide pre-S (12-32)
23 was prepared according to Example 8. Their sequences are:
24 MQWNSTAFHQTLQDPRVRGLYLPAGG (pre-S (120-145)) and
25 MGTNLSVPNPLGFFPDHQLDP (pre-S (12-32)). For immunization,
26 the peptides were used in free form, employing alum or
27 Freund's adjuvant, or linked to carriers, i.e., keyhole
28 lympet hemocyanin (KLH) and cross-linked liposomes,
29 respectively. The liposomes were prepared as described in
30

1 Example 7.

2 The best results were obtained with peptides
3 covalently linked to the surface of liposomes (see Fig.10).
4 Immunization with KLH conjugates resulted in a high anti-KLH
5 response (endpoint titers of 1/5,000,000 by radio-
6 immunoassay), apparently causing low booster responses to
7 the peptides. On the other hand, much lower antibody
8 responses (approximately $1/10^3$) to RAT groups were detected,
9 when RAT-containing liposomes were used as carriers.
10 Antibodies to liposomes (lacking RAT) were undetectable.
11 This suggests that liposomes are the carrier of choice for
12 immunization with synthetic peptides.
13

14 Example 12

15 To establish whether or not antigenic determinants
16 corresponding to pre-S gene coded sequences are
17 preferentially present on HBV particles, the reaction of
18 antisera raised against HBV particles with the two synthetic
19 peptides analogues of the pre-S protein was tested. The
20 maximum dilutions of this antiserum at which antibodies
21 reacting with the synthetic peptides were still detectable
22 were: approximately 1/62,500 ($1/2 \times 10^6$ with tests utilizing
23 ^{125}I -labeled protein A instead of labeled second
24 antibodies), and approximately 1/2,560 for peptides
25 pre-S(120-145) and pre-S(12-32), respectively (see Fig. 11).
26 The antiserum (adsorbed on HBsAg-Sepharose to remove
27 antibodies to S-protein) did not react with synthetic
28 peptide analogues of the S-protein, peptide (309-329) of the
29 env gene product (S(135-155)), peptide (222-239) of the env
30 gene product (S(48-65)) and peptide (243-253) of the env

1 gene product (S(69-79)) and was, therefore, specific for
2 pre-S gene coded sequences. In comparison, the dilution
3 endpoints of antisera prepared against the homologous
4 peptides were approximately 1/300,000 and approximately
5 1/80,000 for anti-pre-S(120-145) (see Fig. 11) and
6 anti-pre-S(12-32) (data not shown), respectively.

7 The synthetic peptides were recognized also by
8 antibodies (IgG and IgM) in sera of individuals who had just
9 recovered from acute hepatitis B, and by rabbit antibodies
10 against a fusion protein between chloramphenicol
11 acetyltransferase and a portion of pre-S protein expressed
12 in *E. coli* (see Fig. 11).

13 On the other hand, humans vaccinated with
14 pepsin-treated HBsAg (M.R. Hilleman, E.B. Buynak, W.J.
15 McAleer, A.A. McLean, P.J. Provost, A.A. Tytell, in Viral
16 Hepatitis, 1981 International Symposium, W. Szmuness, H.J.
17 Alter, J.E. Maynard, Eds. (Franklin Institute Press,
18 Philadelphia, PA, 1982), pp. 385-397) or with HBsAg produce
19 in yeast (devoid of pre-S gene coded sequences; W.J.
20 McAleer, E.B. Buynak, R.F. Maigetter, D.E. Wambler, W.J.
21 Milbur, M.R. Hilleman, Nature (London), 307, 178 (1984)) did
22 not develop detectable antibodies recognizing either of the
23 two synthetic peptides. On the other hand, 7 out of 12
24 individuals who received a vaccine consisting of intact
25 HBsAg developed these antibodies.

26 Example 13

27
28 Quantitative aspects of the immunological cross-
29 reactivity between pre-S gene coded sequences exposed on HB'
30

0154902

1 particles (or on HBsAg) and the synthetic peptide analogues
2 were tested. The peptides were conjugated to
3 β -galactosidase, and the inhibitory effect of free peptides,
4 HBV and HBsAg, respectively, on the formation of immune
5 complexes containing the enzyme-conjugated peptide was
6 studied. Results shown in Fig. 12 indicate that HBV. at
7 sufficient concentrations, inhibited completely the reaction
8 between anti-pre-S(120-145) and pre-S(120-145)-
9 β -galactosidase. HBsAg had $< 1/5$ of the inhibitory activity
10 corresponding to HBV. The inhibitory activity of
11 pepsin-treated HBsAg was $< 1/1,000$ of the activity
12 corresponding to intact HBsAg. These results indicate the
13 absence in the anti-pre-S(120-145) serum of a subpopulation
14 of antibodies which recognize the synthetic peptide but not
15 the native protein. Such antibody subpopulations are
16 observed in many other antisera raised against synthetic
17 peptide analogues of viral proteins. The concentration of
18 free peptide sufficient for approximately 50% inhibition of
19 the reaction of pre-S(120-145)- β -galactosidase with
20 anti-pre-S(120-145) is approximately 1/100 of that for HBV
21 on a weight basis (see Fig. 11). However, since the
22 molecular weight of pre-S(120-145) is approximately 3 kD and
23 the molecular weight of HBV protein components reacting with
24 anti-pre-S(120-145) (representing a minor ($< 20\%$) portion of
25 the total HBV mass) is between approximately 33 and
26 approximately 67 kD, the molar concentrations of HBV and
27 pre-S(120-145) required for this degree of inhibition are
28 approximately the same. This indicates that the antigenic
29 determinants on the peptide analogue and on the
30

1 corresponding segment of the HBV envelope protein(s) are
2 structurally closely related.
3

4 Example 14

5 A peptide pre-S (94-117) (subtype adw₂) was
6 synthesized according to the procedure described hereinabove
7 in Example 2.
8

9 Example 15

10 A rabbit was immunized with the peptide pre-S
11 (94-117) prepared according to Example 14 and linked to a
12 carrier according to the procedure of Example 7. Such
13 immunization was conducted according to the procedure
14 described hereinabove for Example 3 and was found to produce
15 antibodies in the serum of the rabbit so inoculated.
16 However, the antibody titers were substantially less than
17 those observed for the use of pre-S (120-145) and pre-S
18 (12-32).
19

20 Example 16

21 A peptide pre-S (153-171) (subtype adw₂) was
22 synthesized according to the procedure described hereinabove
23 in Example 2.
24

25 Example 17

26 A rabbit was immunized with the peptide pre-S
27 (153-171) prepared according to Example 16 and linked to a
28 carrier according to the procedure of Example 7. Such
29 immunization was conducted according to the procedure
30

0154902

1 described hereinabove for Example 3 and was found to produce
2 antibodies in the serum of the rabbit so inoculated.
3 However, the antibody titers were substantially less than
4 those observed for the use of pre-S (120-145) and pre-S
5 (12-32).
6

7 Example 18

8 A peptide pre-S (1-21) (subtype adw₂) was
9 synthesized according to the procedure described hereinabove
10 in Example 2.
11

12 Example 19

13 A rabbit was immunized with the peptide pre-S
14 (1-21) prepared according to Example 18 and linked to a
15 carrier according to the procedure of Example 7. Such
16 immunization was conducted according to the procedure
17 described hereinabove for Example 3 and was found to produce
18 antibodies in the serum of the rabbit so inoculated.
19 However, the antibody titers were substantially less than
20 those observed for the use of pre-S (120-145) and pre-S
21 (12-32).
22

23 Example 20

24 A peptide pre-S (32-53) (subtype adw₂) was
25 synthesized according to the procedure described hereinabove
26 in Example 2.
27
28
29
30

Example 21

A rabbit was immunized with the peptide pre-S (32-53) prepared according to Example 20 and linked to a carrier according to the procedure of Example 7. Such immunization was conducted according to the procedure described hereinabove for Example 3 and was found to produce antibodies in the serum of the rabbit so inoculated. However, the antibody titers were substantially less than those observed for the use of pre-S (120-145) and pre-S (12-32).

Example 22

A peptide pre-S (57-73) (subtype adw₂) was synthesized according to the procedure described hereinabove in Example 2.

Example 23

A rabbit was immunized with the peptide pre-S (57-73) prepared according to Example 22 and linked to a carrier according to the procedure of Example 7. Such immunization was conducted according to the procedure described hereinabove for Example 3 and was found to produce antibodies in the serum of the rabbit so inoculated. However, the antibody titers were substantially less than those observed for the use of pre-S (120-145) and pre-S (12-32).

1 Example 24

2 Detection of anti-pre-S protein antibodies in
3 human sera using synthetic peptides.

4 As discussed above, antibodies recognizing
5 synthetic peptide analogues of the pre-S protein were
6 detected in sera of humans during recovery from hepatitis B
7 (Fig. 11). The time course of development of antibodies
8 recognizing pre-S(120-145) in a selected patient is shown in
9 Fig. 13.

10 Anti-pre-S protein antibodies are detected in
11 human sera early during acute hepatitis type B. IgM
12 antibodies recognizing the peptides were detected during
13 HBsAg antigenemia before antibodies to the S-protein
14 (anti-HBs) or to hepatitis B core antigen (anti-HBc) were
15 detectable. After development of the latter two antibodies,
16 the level of antibodies with anti-pre-S specificity
17 declined. Variations of this pattern of anti-pre-S
18 development among patients with hepatitis B were observed.
19 In some cases, antibodies recognizing the synthetic peptides
20 were present even before HBsAg was detected in plasma, or
21 when HBsAg never appeared in blood and the only marker for
22 hepatitis B was anti-HBc and later anti-HBs.

23 Antibodies to pre-S(120-145) were measured by RIA.
24 Similar results were obtained by assaying antibodies to
25 pre-S(12-32). HBsAg, anti-HBs and antibodies to hepatitis B
26 core antigen (anti-HBc) were assayed using commercial test
27 kits (Abbot Laboratories, North Chicago, Illinois). The
28 broken line at the end of bars corresponding to the
29 different markers of HBV infection indicates positivity at
30

1 the termination of surveillance. Antibody titers represent
2 the highest dilution of serum at which radioactivity counts
3 corresponding to the specimens divided by counts
4 corresponding to equally diluted control serum were ≥ 2.1 .

5 Humans vaccinated with pepsin-treated HBsAg
6 (Hilleman, M.R., Buynak, E.B., McAleer, W.J., McLean, A.A.,
7 Provost, P.J. & Tytell, A.A. in Viral Hepatitis, 1981
8 International Symposium (eds. Szmuness, W., Alter, H.J. &
9 Maynard, J.E.) 385-397 (Franklin Institute Press,
10 Philadelphia, PA, 1982)), (pepsin treatment removes all
11 anti-pre-S(120-145) reactive material), or with HBsAg
12 produced in yeast (devoid of pre-S gene coded sequences
13 (McAleer, W.J. Buynak, E.B. Maigetter, R.Z., Wambler, D.E.,
14 Miller, W.J., Hillemann, M.R. Nature, (London), 307, 178-180
15 (1984); did not develop detectable antibodies recognizing
16 either of the two synthetic peptides. On the other hand, 7
17 out of 12 individuals who received a vaccine consisting of
18 intact HBsAg (McAuliffe, V.J., Purcell, R.H., Gerin, J.L. &
19 Tyeryar, F.J. in Viral Hepatitis (eds Szmuness, W., Alter,
20 H.J. & Maynard, J.E.) 425-435, Franklin Institute Press,
21 Philadelphia, PA) developed these antibodies. These 7
22 individuals also had the highest antibody response to the
23 S-protein, as measured by the AUSAB test (Abbott),
24 suggesting that a lack of detectable response to the pre-S
25 protein was due to the sensitivity limits of the test. In
26 this respect, it is of importance that the hepatitis B
27 vaccine heretofore used, the production of which involves
28 pepsin treatment of HBcAg, although highly efficient in
29 apparently healthy individuals, has had low immunogenicity
30

1 and no protective effect in hemodialysis patients (Stevens,
2 C.E., Alter, H.J., Taylor, P.E., Zang, E.A., Harley, E.J. &
3 Szmuness, W., N. Engl. J. Med., 311, 496-501 (1984)). Other
4 vaccines produced without pepsin treatment do not seem to
5 have this defect (Desmyter, J. in Viral Hepatitis and Liver
6 Disease (eds Vyas, G.N., Dienstag, J.L. & Hoofnagle, J.), in
7 press Grune and Stratton, Orlando, Fl. 1984).
8

9 Example 25

10 RIA Tests of Preparations Containing HBV-specific
11 proteins

12 Antibodies to the S-protein were removed from
13 rabbit anti-serum against HBV particles by affinity
14 chromatography (Neurath, A.R., Trepo, C., Chen, M., Prince,
15 A.M., J. Gen. Virol., 30, 277-285 (1976) - See Fig. 14. The
16 tested antigens were: HBV particles and tubular forms (●,
17 Δ); approximately 20 nm spherical particles of HBsAg isolated
18 from plasma (○, Δ); and the latter particles treated with
19 pepsin (1 mg/ml HBsAg, 50ug/ml pepsin in 0.1 M glycine-HCl,
20 pH 2.2, 2 hours at 37°C) (□). The RIA tests were performed
21 as described in Neurath; A.R., Kent, S.B.H., Strick, N.,
22 Science, 224, 392-395 (1984). The concentration of HBsAg
23 S-protein was adjusted to the same level in all preparations
24 tested as based on RIA tests (AUSRIA, Abbot Laboratories).
25 HBV particles (contaminated with tubular forms of HBsAg)
26 were concentrated from serum approximately 100x by
27 centrifugation for 4 hours at 25,000 rpm in a Spinco 35
28 rotor. The concentrate (2 ml) was layered over a
29 discontinuous gradient consisting of 11 ml of each 20, 10
30

1 and 5% sucrose (w/w) in 0.14 M NaCl-0.01 M Tris-0.02% NaN₃,
2 pH 7.2 (TS) and centrifuged for 16 hours at 25,000 rpm in a
3 Spinco rotor SW 27. The final pellet was resuspended in TS.

4 HBV particles were recognized much more
5 efficiently than purified approximately 22 nm spherical
6 particles in RIA tests based on polystyrene beads coated
7 with either anti-pre-S(120-145) or with rabbit antibodies to
8 HBV particles. Treatment of HBsAg with pepsin, a step used
9 in preparing some current hepatitis B vaccines, resulted in
10 an approximately 10³-fold decrease in reactivity with
11 anti-pre-S(120-145). HBsAg from vaccines derived either from
12 infected plasma (Hilleman, M.R., et al, 1982) supra), or
13 produced in yeast McAleer et al (1984), supra), had \leq
14 1/5,000 of the reactivity of intact HBsAg in these tests.

15 In reverse tests, beads coated with HBsAg, with
16 HBV particles, with pepsin-treated HBsAg, or with HBsAg
17 corresponding to the vaccines mentioned above were utilized.
18 IgG antibodies (from different rabbit antisera to pre-S
19 sequences) reacting with the beads were assayed based on the
20 subsequent attachment of labeled anti-rabbit IgG. Positive
21 results using anti-pre-S(120-145) were obtained only with
22 beads coated with intact HBsAg or with HBV particles.
23 Anti-pre-S(12-32) reacted exclusively with HBV-coated beads.

24 Example 26

25 Involvement of pre-S Gene Coded HBV Domains In 26 Attachment to Cell Receptors

27 It has been suggested that the 55 C-terminal amino
28 acids of the pre-S protein mediate the attachment of HBsAg
29 to human albumin polymerized by glutaraldehyde (PHSA) and
30

0154902

1 that this attachment plays an essential role in the in vivo
2 adsorption of HBV to hepatocytes (Machida, A. et al,
3 Gastroenterology, 86, 910-918, (1984); Machida, A. et al,
4 Gastroenterology, 85, 268-274, (1983). However, there is no
5 compelling evidence to support the role of the pHSA-HBV
6 interaction in infection of liver cells by HBV. In
7 addition, both HBsAg containing or lacking these 55 amino
8 acid residues react with pHSA (Fig. 15), albeit the reaction
9 is enhanced by the presence of the pre-S gene coded
10 sequences. The RIA tests involved in Fig. 15 were conducted
11 as described in Neurath, A.R., Strick, N. Intervirology, 11,
12 128-132 (1979).

13 To explore directly the reaction of HBsAg with
14 liver cells, an assay system based on the attachment of
15 liver cells to insolubilized HBsAg was developed.

16 HBsAg (HBV) was attached to
17 N-N'-p-phenylenedimaleimide-derivatized sulfhydryl cellulose
18 under conditions described for linking of pre-S(120-145), as
19 described above. About 4 mg of HBsAg was linked to 1 g of
20 the cellulose derivative. A control cellulose derivative
21 was prepared by linking bovine serum albumin to the
22 activated matrix. Forty mg of the cellulose derivative
23 suspended in TS containing 10 mg/ml of bovine serum albumin
24 (TS-BSA) were mixed with approximately 2×10^6 washed Hep G2
25 human hepatoma cells (see Aden, D.P., Fogel, A., Plotkin,
26 S., Damjanov, J., Knowles, B.B., Nature (London), 282,
27 615-617 (1979) suspended in TS-BSA and incubated for 30 min
28 at 37°C, followed by 1 hour at 4°C. HeLa cells and Clone 9
29 normal rat liver cells (American Type Culture Collection)
30

95

1 were used as controls. The cell-cellulose mixtures were
2 layered on top of 1 ml of 33% (w/w) Hypaque and centrifuged
3 for 2 minutes at 3,000 rpm. The cellulose derivative with
4 attached cells pelleted under these conditions. Unattached
5 cells recovered from the Hypaque-TS-BSA interphase were
6 diluted 5-fold in TS-BSA and pelleted by centrifugation.
7 The relative proportion of adsorbed and unadsorbed cells was
8 determined by measurement of lactate dehydrogenase (LDH)
9 activity in appropriate aliquots of cell lysates obtained
10 after exposure to the detergent Triton X-100 (5 mg/ml in
11 H₂O). LDH activity was determined using diagnostic kit No.
12 500 (Sigma).

13 Approximately 80 to 95% of human hepatoma Hep G2
14 cells (Aden, D.P. supra) attached to immobilized HBsAg in
15 this assay. The attachment of control cells (HeLa, rat
16 hepatocytes) was in the range of 10 to 20%. About 10% of
17 Hep G2 cells attached to control cellulose. In the presence
18 of anti-pre-S(120-145) and anti-pre-S(12-32) IgG (15 mg/ml),
19 the adsorption of Hep G2 cells to HBsAg-cellulose decreased
20 to 60 and 30%, respectively. A mixture of both antibodies
21 (7.5 mg/ml of IgG each) caused a decrease of cell adsorption
22 to 20%, indistinguishable from background levels.

23 Normal rabbit IgG, as well as antibodies to the
24 S-protein (elicited by immunization with pepsin-treated
25 HBsAg), failed to diminish the cell attachment, despite high
26 levels of anti-HBs present in this serum (positive at a 10⁻⁶
27 dilution in the AUSAB test).
28

29 It will be appreciated that the instant
30 specification and claims are set forth by way of

0154902

1 illustration and not limitation and that various
2 modifications and changes may be made without departing from
3 the spirit and scope of the present invention.
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30

1 WHAT IS CLAIMED IS:
2

3 1. A hepatitis B peptide immunogen comprising
4 a peptide containing an amino acid chain
5 corresponding to at least six consecutive amino acids within
6 the pre-S gene coded region of the envelope of HBV, said
7 peptide immunogen free of an amino acid sequence
8 corresponding to the naturally occurring envelope proteins
9 of hepatitis B virus.

10 2. A hepatitis B peptide immunogen according to
11 claim 1, wherein said chain of amino acids is between
12 sequence position pre-S 120 and pre-S 174.

13 3. A hepatitis B peptide immunogen according to
14 claim 2, wherein said chain of amino acids includes
15 N-terminal methionine at sequence position pre-S 120.

16 4. A hepatitis B peptide immunogen according to
17 claim 1, wherein said chain of amino acids is between
18 sequence position pre-S 1 and pre-S 120.

19 5. A hepatitis B peptide immunogen according to
20 claim 1, wherein said peptide contains a chain of at least
21 10 amino acids.

22 6. A hepatitis B peptide immunogen according to
23 claim 1, wherein said peptide contains a chain of at least
24 15 amino acids.

25 7. A hepatitis B peptide immunogen according to
26 claim 1, wherein said peptide contains a chain of at least
27 20 amino acids.
28
29
30

0154902

1 8. A hepatitis B peptide immunogen according to
2 claim 1, wherein said peptide contains a chain of at least
3 26 amino acids.

4 9. A hepatitis B peptide immunogen according to
5 claim 8, wherein said chain is between and including
6 sequence positions pre-S 120 and pre-S 174.

7 10. A hepatitis B peptide immunogen according to
8 claim 9, wherein said chain includes N-terminal methionine
9 at sequence position pre-S 120.

10 11. A hepatitis B peptide immunogen according to
11 claim 1, wherein said chain is between and including
12 sequence position pre-S 15 and pre-S 120.

13 12. A hepatitis B peptide immunogen according to
14 claim 1, wherein said chain is between and including
15 sequence position pre-S 15 and pre-S 55.

16 13. A hepatitis B peptide immunogen according to
17 claim 1, wherein said chain is between and including
18 sequence position pre-S 90 and pre-S 120.

19 14. A hepatitis B peptide immunogen according to
20 claim 1, wherein said chain is between and including
21 sequence position pre-S 10 and pre-S 40.

22 15. A hepatitis B peptide immunogen according to
23 claim 1, wherein said chain corresponds to amino acids in
24 the ayw subtype of the pre-S region.

25 16. A hepatitis B peptide immunogen according to
26 claim 1, wherein said chain corresponds to amino acids in
27 the adyw subtype of the pre-S region.
28

29

30

1 17. A hepatitis B peptide immunogen according to
2 claim 1, wherein said chain corresponds to amino acids in
3 the adw2 subtype of the pre-S region.

4 18. A hepatitis B peptide immunogen according to
5 claim 1, wherein said chain corresponds to amino acid in the
6 adw subtype of the pre-S region.

7 19. A hepatitis B peptide immunogen according to
8 claim 1, wherein said chain corresponds to amino acids in
9 the adr subtype of the pre-S region.

10 20. A hepatitis B peptide immunogen according to
11 claim 1, wherein said peptide immunogen is free of any serum
12 proteins.

13 21. A hepatitis B peptide immunogen according to
14 claim 1, wherein said chain is MQWNSTAFHQTLQDPRVRGLYLPAGG.

15 22. A hepatitis B peptide immunogen according to
16 claim 1, wherein said chain is MGTNLSVPNPLGFFPDHQLDP.

17 23. A hepatitis B peptide immunogen according to
18 claim 1, wherein said chain is PAFGANSNNPDWFNPNVKDDWP.

19 24. A hepatitis B peptide immunogen according to
20 claim 1, wherein said chain is PQAMQWNSTAFHQTLQDP.

21 25. A hepatitis B peptide immunogen according to
22 claim 1, wherein said chain is PASTNRQSGRQPTPISPPLRDSHP.

23 26. A hepatitis B peptide immunogen according to
24 claim 1, wherein said chain is PAPNIASHISSISARTGDP.

25 27. A hepatitis B peptide immunogen according to
26 claim 1, wherein said chain is MGGWSSKPRKGMGTNLSVNP.

27 28. A hepatitis B peptide immunogen according to
28 claim 1, wherein said chain is PAFGANSNNPDWFNPNVKDDWP.
29
30

0154902

1 29. A hepatitis B peptide immunogen according to
2 claim 1, wherein said chain is QVGVGAFGPRLTPPHGG.

3 30. A hepatitis B peptide immunogen according to
4 claim 1, wherein said chain is MGGWSSKPRKG.

5 31. A hepatitis B peptide immunogen according to
6 claim 1, wherein said chain is MGGWSSKPRQG.

7 32. A hepatitis B peptide immunogen according to
8 claim 1, wherein said peptide immunogen is free of an amino
9 acid sequence corresponding to the entire S gene coded
10 region of the env gene product of hepatitis B virus.

11 33. A hepatitis B peptide immunogen according to
12 claim 1, wherein said peptide has no more than 100 amino
13 acids.

14 34. A hepatitis B peptide immunogen according to
15 claim 1, wherein said peptide has no more than 40 amino
16 acids.

17 35. A hepatitis B peptide immunogen according to
18 claim 1, wherein said peptide has no more than 30 amino
19 acids.

20 36. A hepatitis B peptide immunogen according to
21 claim 1, wherein said peptide is capable of forming
22 neutralizing antibodies to hepatitis B virus in a humans.

23 37. A hepatitis B peptide immunogen according to
24 claim 1, wherein said peptide is linked to a carrier.

25 38. A hepatitis B peptide immunogen according to
26 claim 37, wherein said peptide is covalently linked to a
27 carrier.
28
29
30

39. A hepatitis B peptide immunogen according to claim 38, wherein said peptide is covalently linked to a lipid vesicle carrier.

40. A hepatitis B peptide immunogen according to claim 39, wherein said lipid vesicle is stabilized by cross-linking.

41. A hepatitis B peptide immunogen according to claim 1, wherein said chain is pre-S(120-145).

42. A hepatitis B peptide immunogen according to claim 1, wherein said chain is pre-S(12-32).

43. A hepatitis B peptide immunogen according to claim 1, wherein said chain is pre-S(117-134).

44. A hepatitis B peptide immunogen according to claim 1, wherein said chain is pre-S(94-117).

45. A hepatitis B peptide immunogen according to claim 1, wherein said chain is pre-S(153-171).

46. A hepatitis B peptide immunogen according to claim 1, wherein said chain is pre-S(1-21).

47. A hepatitis B peptide immunogen according to claim 1, wherein said chain is pre-S(57-73).

48. A carrier for a peptide comprising a lipid vesicle stabilized by cross-linking and having covalently bonded active sites on the outer surface thereof to bind the peptide to the outer surface of the carrier.

49. A carrier according to claim 48, wherein said active sites are selected from the group consisting of -COOH, -CHO, -NH₂ and -SH.

50. A carrier according to claim 49, wherein said lipid vesicle contains an amino moiety selected from the

0154902

1 group consisting of aminoalkane, diaminoalkane, aminoalkene
2 and diaminoalkene having 10 to 18 carbon atoms and said
3 lipid vesicle is stabilized by contacting said lipid vesicle
4 with a polyaldehyde.

5 51. A carrier according to claim 50, wherein said
6 polyaldehyde is a bifunctional aldehyde.

7 52. A carrier according to claim 51, wherein said
8 bisaldehyde is glutaraldehyde.

9 53. A carrier according to claim 50, wherein said
10 amino moiety is stearylamine.

11 54. A carrier according to claim 52, wherein said
12 amino moiety is stearylamine.

13 55. A carrier according to claim 48, wherein said
14 lipid vesicle contains fatty acids having 12 to 18 carbon
15 atoms and said lipid vesicle is stabilized with a
16 carbodiimide.

17 56. A carrier according to claim 55, wherein said
18 fatty acid is stearic acid and said carbodiimide is
19 N-ethyl-N' (dimethyl-aminopropyl)-carbodiimide.

20 57. A carrier according to claim 48, wherein said
21 lipid vesicle contains fatty acid aldehyde.

22 58. A peptide linked to a carrier comprising a
23 peptide having -SH groups and a carrier comprising a liquid
24 vesicle containing an amino moiety selected from the group
25 consisting of aminoalkane, diaminoalkane, aminoalkene,
26 diaminoalkene having 10 to 18 carbon atoms activated by a
27 polyaldehyde and further activated by cysteine.

28 59. A peptide linked to a carrier according to
29 claim 58, wherein said -SH groups are supplied by cysteine.
30

1 60. A peptide linked to a carrier comprising a
2 peptide having -SH groups and a carrier comprising a lipid
3 vesicle containing fatty acid mercaptan.

4 61. A peptide linked to a carrier according to
5 claim 60, wherein said fatty acid mercaptan is
6 octadecanethiol.

7 62. A peptide linked to a carrier according to
8 claim 60, wherein said fatty acid mercaptan contains lipid
9 vesicle activated with a dimaleimide.

10 63. A peptide linked to a carrier according to
11 claim 52, wherein said dimaleimide is N-N'-phenylanedi-
12 maleimide.

13 64. A peptide linked to a carrier comprising a
14 peptide activated by a carbodiimide and a carrier comprising
15 a lipid vesicle containing an amino moiety selected from the
16 group consisting of aminoalkane, diaminoalkane, aminoalkene,
17 diaminoalkene having 10 to 18 carbon atoms.

18 65. A peptide linked to a carrier according to
19 claim 64, wherein said amino moiety is stearylamine.

20 66. A peptide linked to a carrier according to
21 claim 64, wherein said carbodiimide is
22 N-ethyl-N'(dimethylaminopropyl)-carbodiimide.

23 67. A peptide linked to a carrier comprising a
24 peptide activated by a carbodiimide and a carrier comprising
25 a lipid vesicle stabilized by a polyaldehyde and further
26 derivatized by reaction with a water-soluble diaminoalkane.

27 68. A peptide linked to a carrier according to
28 claim 67, wherein said carbodiimide is N-ethyl-N'(di-
29

30

1 methylaminopropyl)-carbodiimide, said polyaldehyde is
2 glucaraldehyde and said diaminoalkane is ethylenediamine.

3 69. A method of forming a carrier comprising
4 contacting a lipid vesicle containing an amino moiety
5 selected from the group consisting of aminoalkane,
6 diaminoalkane, aminoalkene and diaminoalkene having 10 to 18
7 carbon atoms with a polyaldehyde.

8 70. A method according to claim 69, wherein said
9 amino moiety is stearylamine.

10 71. A method according to claim 69, wherein said
11 polyaldehyde is glutaraldehyde.

12 72. A method of forming a carrier comprising
13 contacting a lipid vesicle containing fatty acid having 12
14 to 18 carbon atoms with a carbodiimide.

15 73. A method of forming a carrier according to
16 claim 72, wherein said fatty acid is stearic acid and said
17 carbodiimide is N-ethyl-N'(dimethylaminopropyl)-
18 carbodiimide.

19 74. A method of forming a carrier comprising
20 contacting a lipid vesicle with a fatty acid aldehyde.

21 75. A method of linking a peptide to a carrier
22 comprising contacting a peptide having -SH groups with a
23 carrier comprising a lipid vesicle containing an amino
24 moiety selected from the group consisting of aminoalkane,
25 diaminoalkane, aminoalkene and diaminoalkene having 10 to 18
26 carbon atoms and contacting said lipid vesicle with a
27 polyaldehyde and cysteine.

28 76. A method of linking a peptide to a carrier
29 comprising contacting a peptide having -SH groups with a
30

1 carrier comprising a lipid vesicle having fatty acid
2 mercaptan.

3 77. A method of linking a peptide to a carrier
4 according to claim 76, wherein said fatty acid mercaptan is
5 octadecanediol.

6 78. A method of linking a peptide to a carrier
7 according to claim 77, wherein said fatty acid mercaptan
8 containing lipid vesicle is contacted with a dimaleimide.

9 79. A method according to claim 78, wherein said
10 dimaleimide is N-N'-phenylatedimaleimide.

11 80. A method of linking a peptide to a carrier
12 comprising contacting a peptide, said peptide contacted with
13 a carbodiimide, with a carrier, said carrier comprising an
14 lipid vesicle containing an amino moiety selected from the
15 group consisting of aminoalkane, diaminoalkane, aminoalkene
16 and diaminoalkene having 10 to 18 carbon atoms.

17 81. A method according to claim 80, wherein said
18 amino moiety is stearylamine and said carbodiimide is
19 N-ethyl-N' (dimethylaminopropyl)-carbodiimide.

20 82. A method of linking a peptide to a carrier
21 comprising contacting a peptide activated by a carbodiimide
22 and a carrier comprising a lipid vesicle stabilized by a
23 polyaldehyde and further reacted with a water-soluble
24 diaminoalkane.

25 83. A method of linking a peptide to a carrier
26 according to claim 82, wherein said polyaldehyde is
27 glutaraldehyde and said diaminoalkane is ethylenediamine.

28 84. A peptide comprising an amino acid chain
29 corresponding to at least six consecutive amino acids within
30

1 the pre-S gene coded region or the envelope of HBV, said
2 peptide free of an amino acid sequence corresponding to the
3 naturally occurring envelope proteins of hepatitis B virus.

4 85. A peptide according to claim 84, wherein
5 said chain of amino acids is between sequence position pre-S
6 120 and pre-S 174.

7 86. A peptide according to claim 85, wherein
8 said chain of amino acids includes N-terminal methionine at
9 sequence position pre-S 120.

10 87. A peptide according to claim 84, wherein
11 said chain of amino acids is between sequence position pre-S
12 1 and pre-S 120.

13 88. A peptide according to claim 84, wherein
14 said peptide contains a chain of at least 10 amino acids.

15 89. A peptide according to claim 84, wherein
16 said peptide contains a chain of at least 15 amino acids.

17 90. A peptide according to claim 84, wherein
18 said peptide contains a chain of at least 20 amino acids.

19 91. A peptide according to claim 84, wherein
20 said peptide contains a chain of at least 26 amino acids.

21 92. A peptide according to claim 91, wherein
22 said chain is between and including sequence positions pre-S
23 120 and pre-S 174.

24 93. A peptide according to claim 92, wherein
25 said chain includes N-terminal methionine at sequence
26 position pre-S 120.

27 94. A peptide according to claim 84, wherein
28 said chain is between and including sequence position pre-S
29 15 and pre-S 120.
30

- 1 95. A peptide according to claim 84, wherein
2 said chain is between and including sequence position pre-S
3 15 and pre-S 55.
- 4 96. A peptide according to claim 84, wherein
5 said chain is between and including sequence position pre-S
6 90 and pre-S 120.
- 7 97. A peptide according to claim 84, wherein
8 said chain is between and including sequence position pre-S
9 10 and pre-S 40.
- 10 98. A peptide according to claim 84, wherein
11 said chain corresponds to amino acids in the ayw subtype of
12 the pre-S region.
- 13 99. A peptide according to claim 84, wherein
14 said chain corresponds to amino acids in the adyw subtype of
15 the pre-S region.
- 16 100. A peptide according to claim 84, wherein
17 said chain corresponds to amino acids in the adw2 subtype of
18 the pre-S region.
- 19 101. A peptide according to claim 84, wherein
20 said claim corresponds to amino acids in the adw subtype of
21 the pre-S region.
- 22 102. A peptide according to claim 84, wherein
23 said chain corresponds to amino acids in the adr subtype of
24 the pre-S region.
- 25 103. A peptide according to claim 84, wherein
26 said peptide is MQWNSTAFHQTLQDPRVRGLYLPAGG.
- 27 104. A peptide according to claim 84, wherein
28 said peptide is MGTNLSVPNPLGFFPDHQLDP.
- 29
30

0154902

1 105. A peptide according to claim 84, wherein
2 said peptide is PAFGANSNPWFNPNVKDDWP.

3 106. A peptide according to claim 84, wherein
4 said peptide is PQAMQWNSTAFHQTLQDP.

5 107. A peptide according to claim 84, wherein
6 said peptide is PASTNRQSGRQPTPISPPLRDSHP.

7 108. A peptide according to claim 84, wherein
8 said peptide is PAPNIASHISSISARTGDP.

9 109. A peptide according to claim 84, wherein
10 said peptide is MGGWSSKPRKGMTNLSVPPNP.

11 110. A peptide according to claim 84, wherein
12 said peptide is PAFGANSNNPDWDFNPNVKDDWP.

13 111. A peptide according to claim 84, wherein
14 said peptide is QVGVGAFGPRLTPPHGG.

15 112. A peptide according to claim 84, wherein
16 said peptide is MGGWSSKPRKG.

17 113. A peptide according to claim 84, wherein
18 said peptide is MGGWSSKPRKG.

19 114. A peptide according to claim 84, wherein
20 said peptide is free of an amino acid sequence corresponding
21 to the entire S gene coded region of the surface antigen of
22 hepatitis B virus.

23 115. A peptide according to claim 84, wherein
24 said peptide has no more than 100 amino acids.

25 116. A peptide according to claim 84, wherein
26 said peptide has no more than 40 amino acids.

27 117. A peptide according to claim 84, wherein
28 said peptide has no more than 30 amino acids.
29
30

- 1 118. A peptide according to claim 84, wherein
2 said chain is pre-S(120-145).
3 119. A peptide according to claim 84, wherein
4 said chain is pre-S(12-32).
5 120. A peptide according to claim 84, wherein
6 said chain is pre-S(117-134).
7 121. A peptide according to claim 84, wherein said
8 chain is pre-S(94-117).
9 122. A peptide according to claim 84, wherein
10 said chain is pre-S(153-171).
11 123. A peptide according to claim 84, wherein
12 said chain is pre-S(1-21).
13 124. A peptide according to claim 84, wherein
14 said chain is pre-S(57-73).
15 125. A process for the detection of antigens
16 coded for the pre-S gene in sera of HBV infected animals
17 comprising:
18 (a) coating a solid substrate with
19 antibodies to a peptide having an amino acid chain
20 corresponding to at least six consecutive amino acids within
21 the pre-S gene coded region of the envelope of HBV, said
22 peptide free of an amino acid sequence corresponding to the
23 naturally occurring proteins of HBV;
24 (b) washing the coated substrate;
25 (c) contacting the washed coated substrate
26 with a protein-containing solution;
27 (d) washing the substrate from step c;
28 (e) incubating the substrate from step d
29 with a sample suspected to contain HBV or HBsAg;
30

0154902

1 (f) washing the substrate from step e;
2 (g) adding radiolabeled antibody, said
3 antibody being an antibody to the peptide or HBsAg, to the
4 substrate from step f;
5 (h) incubating the substrate from step g;
6 (i) washing the substrate from step h; and
7 (j) subjecting the substrate of step i to
8 counting in a gamma counter.

9 126. A process for the detection of antigen coded
10 for by the pre-S gene in sera of HBV infected animals
11 according to claim 125, wherein said substrate is
12 polystyrene beads.

13 127. A process for the detection of antigen coded
14 for by the pre-S gene in sera of HBV infected animals
15 according to claim 125, wherein said protein-containing
16 solution contains bovine serum albumin or gelatin.

17 128. A process for the detection of antigens coded
18 for the pre-S gene in sera of HBV infected animals
19 comprising:
20

21 (a) coating a solid substrate with
22 antibodies to a peptide having an amino acid chain
23 corresponding to at least six consecutive amino acids within
24 the pre-S gene coded region of the envelope of HBV, said
25 peptide free of an amino acid sequence corresponding to the
26 naturally occurring proteins of HBV;

27 (b) washing the coated substrate;

28 (c) contacting the washed coated substrate
29 with a protein-containing solution;

30 (d) washing the substrate from step c;

- 1 (e) incubating the substrate from step d
- 2 with a sample suspected to contain HBV or HBsAg;
- 3 (f) washing the substrate from step e;
- 4 (g) adding enzyme labeled antibody, said
- 5 antibody being an antibody to the peptide or HBsAg, to the
- 6 substrate from step f;
- 7 (h) incubating the substrate from step g;
- 8 (i) washing the substrate from step h;
- 9 (j) subjecting the substrate of step i to
- 10 ELISA and comparing the results of said ELISA to ELISA
- 11 results from normal sera utilized as a control.

12 129. A process for the detection of antigens coded
13 for by the pre-S gene in sera of HBV infected animals
14 according to claim 128, wherein said substrate is
15 polystyrene beads.

16 130. A process for the detection of antibodies to
17 the pre-S region of hepatitis B virus comprising:

18 (a) adsorbing on a solid substrate,
19 containing binding sites thereon, a peptide having an amino
20 acid chain corresponding to at least six consecutive amino
21 acids within the pre-S gene coded region of the envelope of
22 HBV, said peptide free of an amino acid sequence
23 corresponding to the naturally occurring envelope proteins
24 of hepatitis B virus,

25 (b) contacting the substrate from step a
26 with a material to saturate the binding sites thereon,

27 (c) washing the substrate from step b,

28
29
30

0154902

1 (d) contacting the substrate from step c
2 with a specimen comprising human sera to form a first
3 resultant mass,
4 (e) incubating the resultant mass of step d,
5 (f) washing the resultant mass of step e,
6 (g) adding radiolabeled antibodies to human
7 IGG or IGM to the resultant mass of step f to form a second
8 resultant mass,
9 (h) subjecting the second resultant mass of
10 step g to counting in a gamma counter,
11 (i) subjecting normal sera utilized as a
12 control to steps a to h and
13 (j) comparing the counts of steps h and i.
14 131. A process according to claim 130, wherein
15 said substrate is polystyrene beads.
16 132. A process for the detection of antibodies to
17 the pre-S region of hepatitis B virus comprising:
18 (a) adsorbing on a solid substrate,
19 containing binding sites thereon, a peptide having an amino
20 acid chain corresponding to at least six consecutive amino
21 acids within the pre-S gene coded region of the envelope of
22 HBV, said peptide free of an amino acid sequence
23 corresponding to the naturally occurring envelope proteins
24 of hepatitis B virus,
25 (b) contacting the substrate from step a
26 with a material to saturate the binding sites thereon,
27 (c) washing the substrate from step b,
28
29
30

- 1 (d) contacting the substrate from step c
2 with a specimen comprising human sera to form a first
3 resultant mass,
4 (e) incubating the resultant mass of step d,
5 (f) washing the resultant mass of step e,
6 (g) adding enzyme labeled antibodies to
7 human IgG or IgM to the resultant mass of step f to form a
8 second resultant mass,
9 (h) subjecting the second resultant mass of
10 step g to ELISA,
11 (i) subjecting a normal sera utilized as a
12 control to ELISA, and
13 (j) comparing the ELISA results from steps h
14 and i.
15
16 133. A process according to claim 132, wherein
17 said substrate is polystyrene beads.
18 134. A diagnostic test kit for detecting antigens
19 coded for the pre-S gene of HBV in a test sample, comprising
20 a. a solid substrate containing protein
21 binding sites thereon, said substrate coated with antibodies
22 to a peptide having an amino acid chain corresponding to at
23 least six consecutive amino acids within the pre-S gene
24 coded region of the envelope of HBV, said peptide free of an
25 amino acid sequence corresponding to the naturally occurring
26 proteins of HBV,
27 b. a protein-containing solution to
28 saturate protein binding sites on the solid substrate, and
29 c. a given amount of radiolabeled or enzyme
30 labelled antibody, said antibody to either the peptide or

0154902

1 HBSAg.

2 135. A diagnostic test kit for detecting
3 antibodies to the pre-S region of hepatitis B virus in a
4 test sample, comprising

5 a. a solid substrate containing protein
6 binding sites thereon, said substrate having adsorbed
7 thereon a peptide having an amino acid chain corresponding
8 to at least six consecutive amino acids within the pre-S
9 gene coded region of the envelope of HBV, said peptide free
10 of an amino acid sequence corresponding to the naturally
11 occurring proteins of HBV, the solid substrate exposed to
12 a protein-containing solution to saturate protein binding
13 sites on the solid substrate, and

14 b. a given amount of radiolabeled or enzyme
15 labelled antibodies to human IgG or IgM.

16 136. A process for detecting antibodies to the
17 pre-S region of hepatitis B virus in a sample which
18 comprises:

19 a) contacting the sample with a solid substrate
20 coated with a non-labelled peptide containing an amino acid
21 chain corresponding to at least six consecutive amino acids
22 within the pre-S gene coded region of the enveloping of HBV,
23 the peptide free of an amino acid sequence corresponding to
24 the naturally occurring envelope proteins of hepatitis B
25 virus, incubating and washing said contacted sample;

26 b) contacting the incubated washed product
27 obtained from step a above with a labelled peptide
28 containing an amino acid chain corresponding to at least six
29 consecutive amino acids within the pre-S gene coded region
30

1 of the envelope of HBV, said peptide free of an amino acid
2 sequence corresponding to the naturally occurring envelope
3 protein of hepatitis B virus, incubating and washing the
4 resultant mass; and

5 c) determining the extent of labelled peptide
6 present in the resultant mass obtained by step b above.

7 137. A process according to claim 136, wherein
8 a solid substrate is rendered substantially free of
9 available protein binding sites.

10 138. A process according to claim 137, wherein
11 the solid substrate is contacted with a protein binding site
12 occupier.

13 139. A process according to claim 139, wherein
14 the occupier is albumin.

15 140. A process for detecting antibodies to the
16 pre-S region of hepatitis B virus in a sample comprising:

17 a) contacting the sample with a solid substrate
18 coated with a non-labelled peptide containing an amino acid
19 chain corresponding to at least six consecutive amino acids
20 within the pre-S gene coded region of the envelope of HBV,
21 the peptide free of an amino acid sequence corresponding to
22 the naturally occurring envelope proteins of hepatitis B
23 virus, incubating and washing said contacted sample;

24 b) contacting the incubated washed product
25 obtained from step a above with labelled antibody to human
26 or animal immunoglobulin product by contact with an
27 immunogen comprising a peptide corresponding to at least six
28 consecutive amino acids within the pre-S gene coded region
29 of the envelope of HBV, said peptide immunogen free of an
30

0154902

1 amino acid sequence corresponding to the naturally occurring
2 envelope proteins of hepatitis B virus, incubating and
3 washing the contacted sample, and

4 c) determining the extent of labelled antibody
5 present in the resultant mass of step b.

6 141. A process for detecting HBV or HBsAg in a
7 sample comprising:

8 a) contacting a first portion of a composition
9 containing an antibody produced by introducing into an
10 animal or human an immunogen comprising a peptide
11 corresponding to at least six consecutive amino acids within
12 the pre-S gene coded region of the envelope of HBV, said
13 peptide immunogen free of an amino acid sequence
14 corresponding to the naturally occurring envelope proteins
15 of hepatitis B virus with a mixture of said sample and said
16 immunogen which has been labelled, incubating and washing
17 said first protein;

18 b) contacting a second portion of said
19 composition containing antibody with the same amount of said
20 labelled immunogen in an antigen free control, incubating
21 and washing said second portion;

22 c) adding the same amount of Staphylococci
23 bearing protein A to each of the compositions of steps a and
24 b above, incubating both of said compositions, centrifuging
25 each of said compositions and separating liquid from the
26 solids therein;

27 d) determining the extent of labelled immunogen
28 in each of the resultant compositions from step c above, and

29 e) comparing the relative amount of labelled
30

1 immunogen in each such that if the activity of the resultant
2 composition containing the first portion is less than the
3 activity for the resultant composition of the second
4 portion, then the sample contains HBV or HBsAg.

5 142. A diagnostic test kit for detecting
6 hepatitis B virus in sera comprising

7 a) a given amount of antibody to a peptide
8 containing an amino acid chain corresponding to at least six
9 consecutive amino acids within the pre-S gene coded region
10 of the envelope of HBV, said peptide being free of an amino
11 acid chain corresponding to the naturally occurring envelope
12 proteins of hepatitis B virus, the antibody being bound to a
13 solid support,

14 b) labelled antibody to the peptide or to
15 hepatitis B virus.
16

17 143. A diagnostic test kit for detecting
18 hepatitis B virus in sera according to claim 142, which
19 further comprises a set of instructions for effecting an
20 immunoassay wherein the effect of formation of an immune
21 complex is revealed by said labelled antibody.

22 144. A diagnostic test kit for detecting
23 hepatitis B virus in sera according to claim 143, wherein
24 said antibody is insolubilized on a water insoluble solid
25 support.

26 145. A diagnostic kit for detecting the presence
27 of antibodies to hepatitis B virus comprising

28 a) a given amount of a peptide containing
29 an amino acid chain corresponding to at least six
30 consecutive amino acids within the pre-S gene coded region

0154902

1 of the envelope of HBV, said peptide being free of an amino
2 acid chain corresponding to the naturally occurring envelope
3 proteins of hepatitis B virus,

4 b) labelled antibodies to human IgG or IgM.

5 146. A diagnostic kit for detecting the presence
6 of antibodies to hepatitis B virus according to claim 145,
7 which further comprises a set of instructions for effecting
8 an immunoassay, wherein the extent of formation of an immune
9 complex is revealed by said labelled antibodies.

10 147. A diagnostic test kit for detecting
11 hepatitis B virus in sera according to claim 145, wherein
12 said peptide is insolubilized upon a water insoluble solid
13 support.

14 148. A process for predicting the outcome of
15 hepatitis B infection which comprises carrying out an
16 immunoassay on serum of a human to detect the presence of an
17 antibody to an antigen coded for by the pre-S gene coded
18 region of the envelope of hepatitis B virus employing the
19 peptide immunogen of claim 1 at regular intervals and
20 evaluating the data.

21 149. A process for determining if a human who has
22 been vaccinated with a vaccine against hepatitis B has
23 become immune to hepatitis B virus which comprises effecting
24 a plurality of immunoassays of serum from such human to
25 determine if there are antibodies in said serum to an
26 antigen coded by the pre-S gene coded region of the envelope
27 of hepatitis B virus employing the peptide immunogen of
28 claim 1, said immunoassays being performed on serum taken
29 from said human at different times.
30

1 150. A method for detecting the presence of
2 hepatitis B virus infection comprising effecting quantative
3 immunoassays on a serum sample taken from a human to
4 determine the amount of antibodies present therein which are
5 antibodies to an antigen coded by the pre-S gene coded
6 region of the envelope of the hepatitis B virus employing
7 the peptide immunogen of claim 1 and comparing the value
8 with a known standard.

9 151. A process for raising antibodies which
10 comprises introducing into an animal the peptide immunogen
11 of claim 1.

12 152. In a process for synthesizing His and Trp
13 containing peptides which includes the steps of

- 14 a. linking a first amino acid containing an
15 alpha-amino protecting group to a resin;
16 b. removal of said alpha-amino protecting group;
17 c. coupling a second amino acid containing an
18 alpha-amino protecting group to said first amino acid;
19 d. repeating steps b and c by coupling further
20 alpha-protected amino acids to produce a desired peptide
21 wherein at least one of said amino acids is His
22 and wherein at least one of said amino acids is Trp and the
23 e. cleaving the peptide from the resin and
24 removing remaining protective groups to said first amino
25 acids,
26

27 wherein the improvement comprises substituting an
28 His(ImDNP) for said His, substituting a Trp(InFormyl) for
29 said Trp, removing said DNP prior to cleavage and said
30 removing of protective group, and removing said Formyl

0154902

1 during said cleavage and said removing of protective group.

2 153. A hepatitis B vaccine comprising

3 a peptide containing an amino acid chain
4 corresponding to at least six consecutive amino acids within
5 the pre-S gene coded region of the envelope of HBV, said
6 vaccine free of an amino acid sequence corresponding to the
7 naturally occurring envelope proteins of hepatitis B virus,
8 and

9 a physiologically acceptable diluent.

10 154. A hepatitis B vaccine according to claim 153,
11 wherein said chain of amino acids is between sequence
12 position pre-S 120 and pre-S 174.

13 155. A hepatitis B vaccine according to claim 154,
14 wherein said chain of amino acids includes N-terminal
15 methionine at sequence position pre-S 120.

16 156. A hepatitis B vaccine according to claim 153,
17 wherein said chain of amino acids is between sequence
18 position pre-S 1 and pre-S 120.

19 157. A hepatitis B vaccine according to claim 153,
20 wherein said peptide contains a chain of at least 10 amino
21 acids.

22 158. A hepatitis B vaccine according to claim 153,
23 wherein said peptide contains a chain of at least 15 amino
24 acids.

25 159. A hepatitis B vaccine according to claim 153,
26 wherein said peptide contains a chain of at least 20 amino
27 acids.

28 160. A hepatitis B vaccine according to claim 153,
29 wherein said peptide contains a chain of at least 26 amino
30

1 acids.

2 161. A hepatitis B vaccine according to claim 160,
3 wherein said chain is between and including sequence
4 positions pre-S 120 and pre-S 174.

5 162. A hepatitis B vaccine according to claim 161,
6 wherein said chain includes N-terminal methionine at
7 sequence position pre-S 120.

8 163. A hepatitis B vaccine according to claim 153,
9 wherein said chain is between and including sequence
10 position pre-S 15 and pre-S 120.

11 164. A hepatitis B vaccine according to claim 153,
12 wherein said chain is between and including sequence
13 position pre-S 15 and pre-S 55.

14 165. A hepatitis B vaccine according to claim 153,
15 wherein said chain is between and including sequence
16 position pre-S 90 and pre-S 120.

17 166. A hepatitis B vaccine according to claim 153,
18 wherein said chain is between and including sequence
19 position pre-S 10 and pre-S 40.

20 167. A hepatitis B vaccine according to claim 153,
21 wherein said chain corresponds to amino acids in the ayw
22 subtype of the pre-S region.

23 168. A hepatitis B vaccine according to claim 153,
24 wherein said chain corresponds to amino acids in the adyw
25 subtype of the pre-S region.

26 169. A hepatitis B vaccine according to claim 153,
27 wherein said chain corresponds to amino acids in the adw2
28 subtype of the pre-S region.
29
30

0154902

1 170. A hepatitis B vaccine according to claim 153,
2 wherein said chain corresponds to amino acid in the adw
3 subtype of the pre-S region.

4 171. A hepatitis B vaccine according to claim 153,
5 wherein said chain corresponds to amino acids in the adr
6 subtype of the pre-S region.

7 172. A hepatitis B vaccine according to claim 153,
8 wherein said vaccine is free of any serum proteins.

9 173. A hepatitis B vaccine according to claim 153,
10 wherein said chain is MQWNSTAFHQTLQDPRVRGLYLPAGG.

11 174. A hepatitis B vaccine according to claim 153,
12 wherein said chain is MGTNLSVPNPLGFFPDHQLDP.

13 175. A hepatitis B vaccine according to claim 153,
14 wherein said chain is PAFGANSNNPDWDFNPVKDDWP.

15 176. A hepatitis B vaccine according to claim 153,
16 wherein said chain is PQAMQWNSTAFHQTLQDP.

17 177. A hepatitis B vaccine according to claim
18 153, wherein said chain is PASTNRQSGRQPTPISPPLRDSHP.

19 178. A hepatitis B vaccine according to claim 153,
20 wherein said chain is PAPNIAASHISSISARTGDP.

21 179. A hepatitis B vaccine according to claim
22 153, wherein said chain is MGGWSSKPRKGMGTNLSVPPNP.

23 180. A hepatitis B vaccine according to claim 153,
24 wherein said chain is PAFGANSNNPDWDFNPVKDDWP.

25 181. A hepatitis B vaccine according to claim 153,
26 wherein said chain is QVGVGAFGPRLTPPHGG.

27 182. A hepatitis B vaccine according to claim 153,
28 wherein said chain is MGGWSSKPRKG.

1 183. A hepatitis B vaccine according to claim 153,
2 wherein said chain is MGGWSSKPRQG.

3 184. A hepatitis B vaccine according to claim
4 153, wherein said vaccine is free of an amino acid sequence
5 corresponding to the entire S gene coded region of the env
6 gene product of hepatitis B virus.

7 185. A hepatitis B vaccine according to claim
8 153, wherein said peptide has no more than 100 amino acids.

9 186. A hepatitis B vaccine according to claim
10 153, wherein said peptide has no more than 40 amino acids.

11 187. A hepatitis B vaccine according to claim
12 153, wherein said peptide has no more than 30 amino acids.

13 188. A hepatitis B vaccine according to claim
14 153, wherein said peptide is capable of forming neutralizing
15 antibodies to hepatitis B virus in a humans.

16 189. A hepatitis B vaccine according to claim 153,
17 wherein said peptide is linked to a carrier.

18 190. A hepatitis B vaccine according to claim 189,
19 wherein said peptide is covalently linked to a carrier.

20 191. A hepatitis B vaccine according to claim
21 190, wherein said peptide is covalently linked to a lipid
22 vesicle carrier.

23 192. A hepatitis B vaccine according to claim 191,
24 wherein said lipid vesicle is stabilized by cross-linking.

25 193. A hepatitis B vaccine according to claim
26 153, wherein said chain is pre-S(120-145).

27 194. A hepatitis B vaccine according to claim
28 153, wherein said chain is pre-S(12-32).
29
30

1 195. A hepatitis B vaccine according to claim
2 153, wherein said chain is pre-S(117-134).

3 196. A hepatitis B vaccine according to claim
4 153, wherein said chain is pre-S(94-117).

5 197. A hepatitis B vaccine according to claim
6 153, wherein said chain is pre-S(153-171).

7 198. A hepatitis B vaccine according to claim
8 153, wherein said chain is pre-S(1-21).

9 199. A hepatitis B vaccine according to claim
10 153, wherein said chain is pre-S(57-73).

11 200. A method of protecting a human against
12 becoming infected with hepatitis B comprising administering
13 to said human an effective dosage of a vaccine according to
14 claim 153.

15 201. A method for detecting the presence of
16 hepatitis B virus infection comprising effecting quantitative
17 immunoassays on a serum sample taken from a human to
18 determine the amount of antigens coded by the pre-S gene
19 coded region of the envelope of the hepatitis B virus
20 employing antibodies to the peptide immunogen of claim 1 and
21 comparing the value with a known standard.
22

23
24
25
26
27
28
29
30

1/14

0154902

FIG. 1
a b

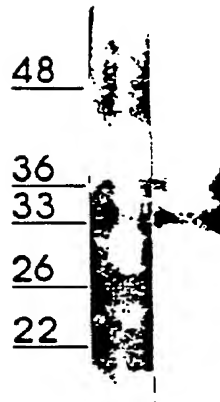


FIG. 6

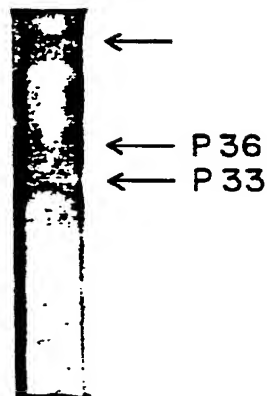


FIG. 2

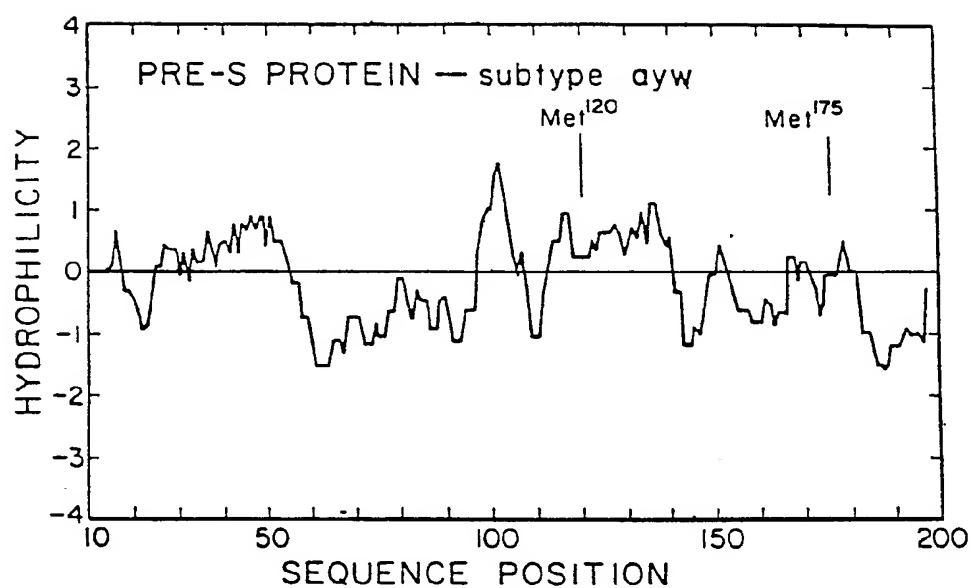
Pre-S protein

	1						2						3						4						5						6																															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40																						
1 ayw	G	H	I	L	G	M	K	I	Y	S	M	G	Q	N	L	S	T	S	N	P	L	G	F	F	P	D	H	Q	L	D	P	A	F	R	A	N	T	A	N	P	D	W	D	F	N	P	H	K	D	T	W	P	D	A	N	K	V	G	A	G	A	
2 adyw	G	H	I	L	G	M	K	S	Y	S	M	G	Q	N	L	S	T	S	N	P	L	G	F	F	P	D	H	Q	L	D	P	A	F	R	A	N	T	A	N	P	D	W	D	F	N	P	H	K	D	T	W	P	D	A	N	K	V	G	A	G	A	
3 adw2	H	G	G	S	S	K	P	R	K	G	M	G	T	N	L	S	V	P	N	P	L	G	F	F	P	D	H	Q	L	D	P	A	F	R	A	N	T	A	N	P	D	W	D	F	N	P	H	K	D	T	W	P	D	A	N	K	V	G	A	G	A	
4 adw	L	G	M	K	S	Y	S	I	R	K	G	M	G	T	N	L	S	V	P	N	P	L	G	F	F	P	D	H	Q	L	D	P	A	F	R	A	N	T	A	N	P	D	W	D	F	N	P	H	K	D	T	W	P	D	A	N	K	V	G	A	G	A
5 adr	H	G	G	S	S	K	P	R	Q	G	H	G	T	N	L	S	V	P	N	P	L	G	F	F	P	D	H	Q	L	D	P	A	F	R	A	N	T	A	N	P	D	W	D	F	N	P	H	K	D	T	W	P	D	A	N	K	V	G	A	G	A	
	H G N L S						M P L G F						P D H Q L D P A F						A N						N P D W D F N P						K D W P						A N V G G A																								
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
													
																																																	

3/14

0154902

FIG. 3



4/14

0154902

FIG. 4A

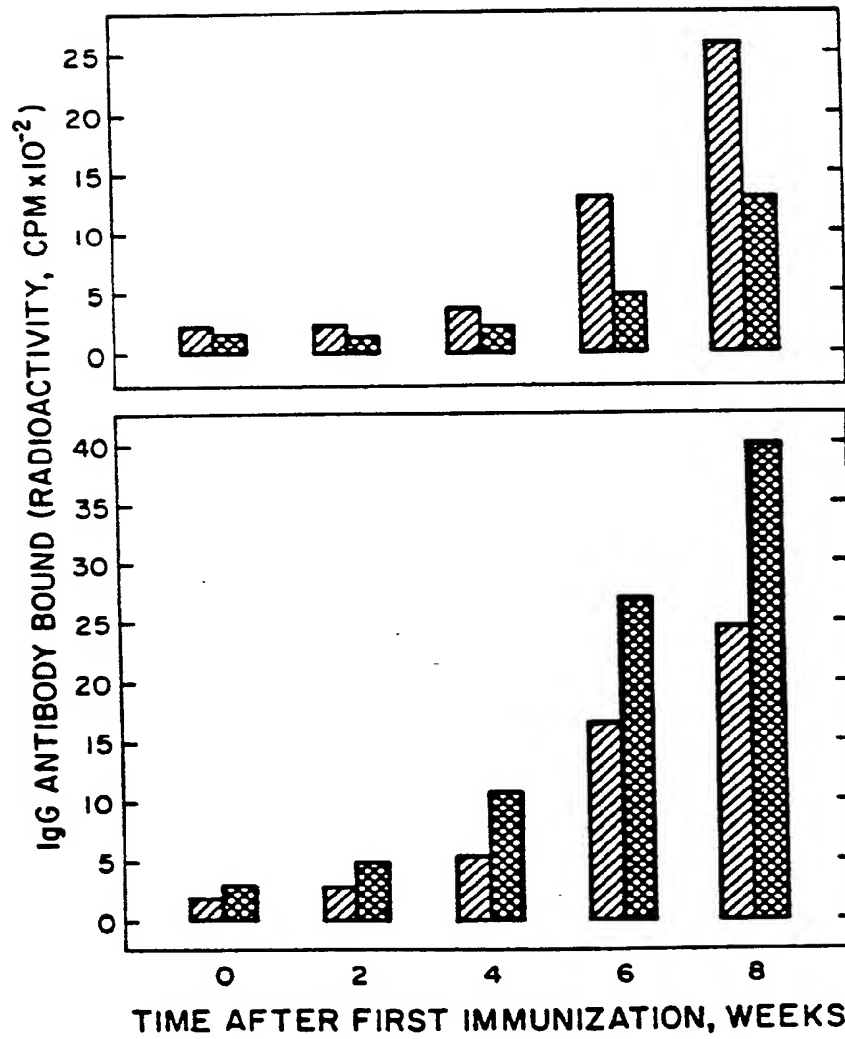
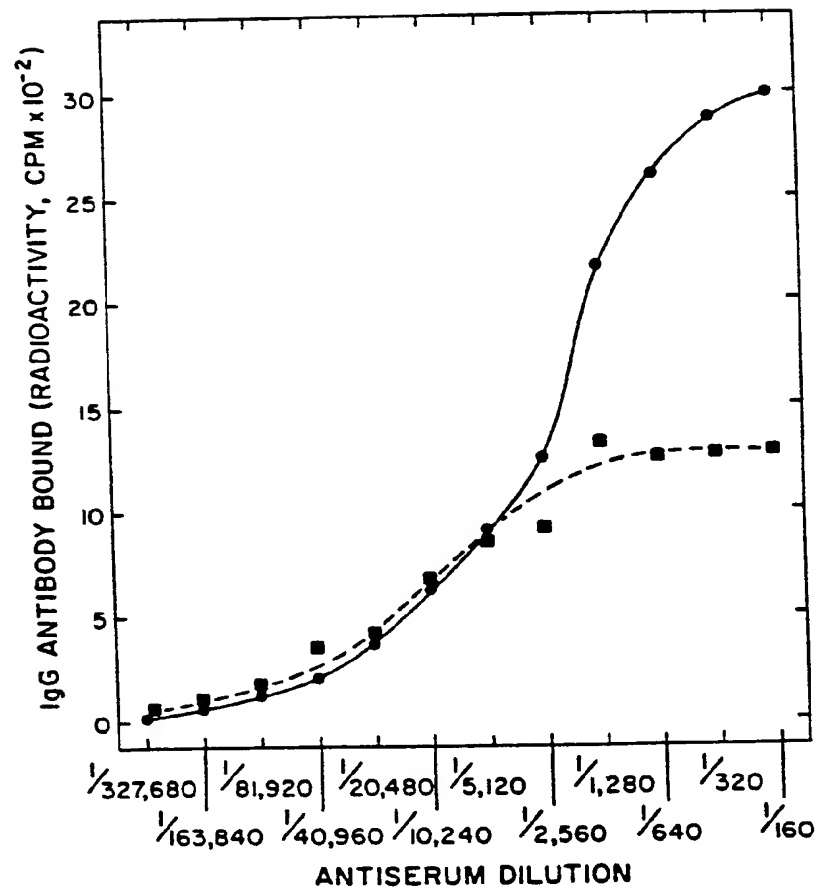


FIG. 4B

5/14

0154902

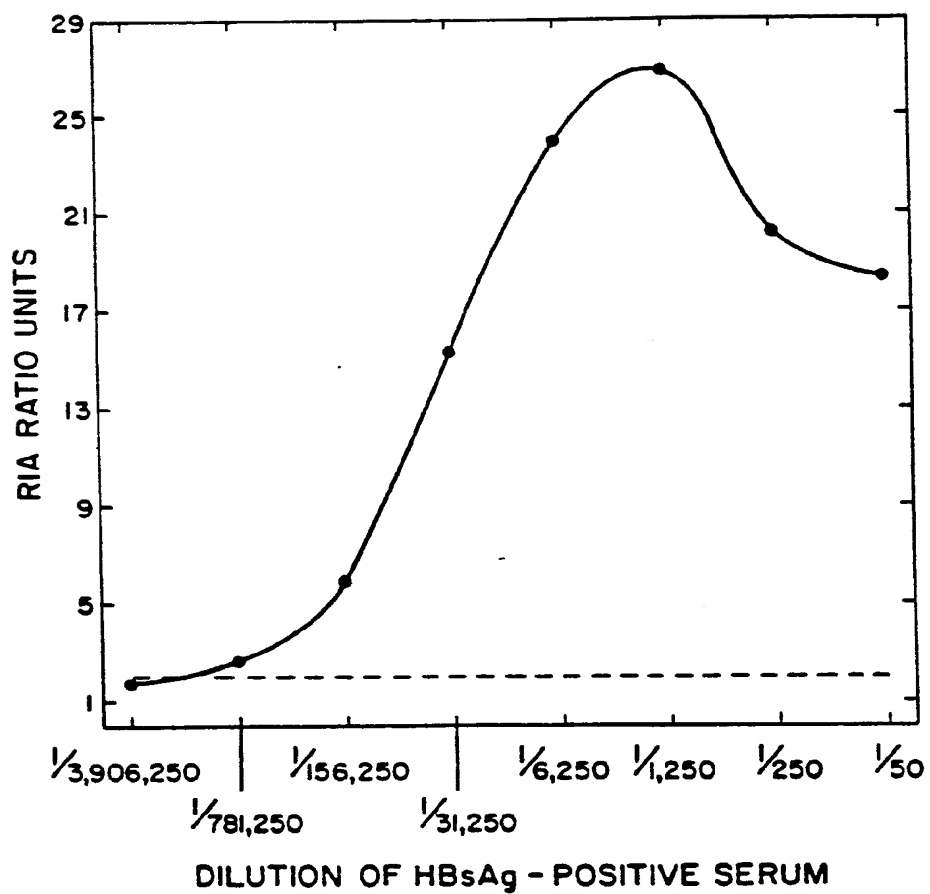
FIG. 5



6/14

0154902

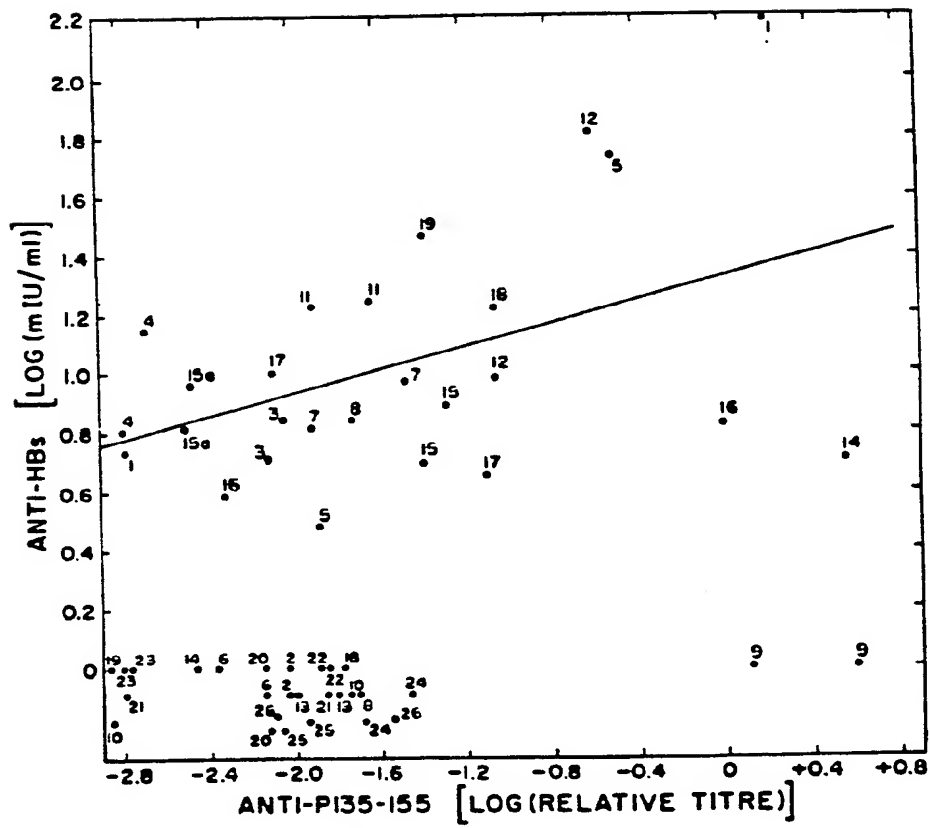
FIG. 7



7/14

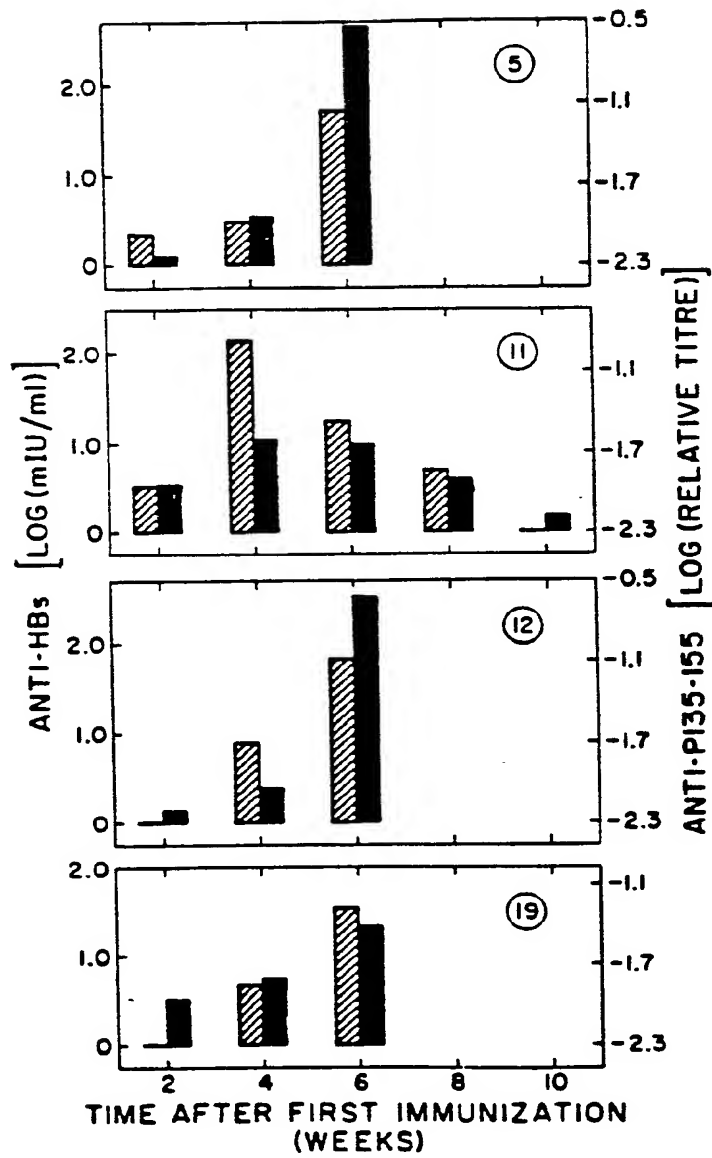
0154902

FIG. 8



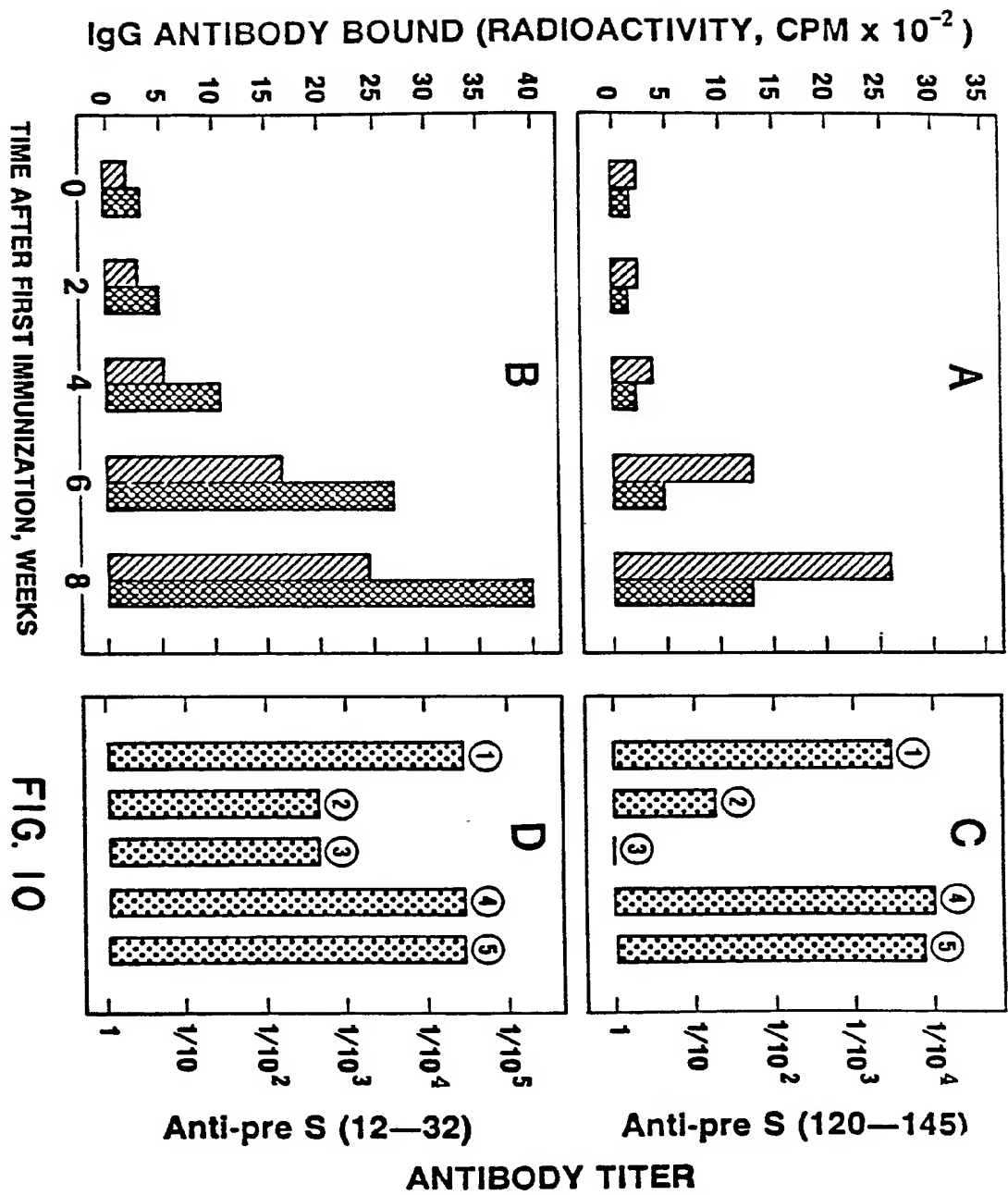
8/14

0154902



9/14

0154902



10/14

0154902

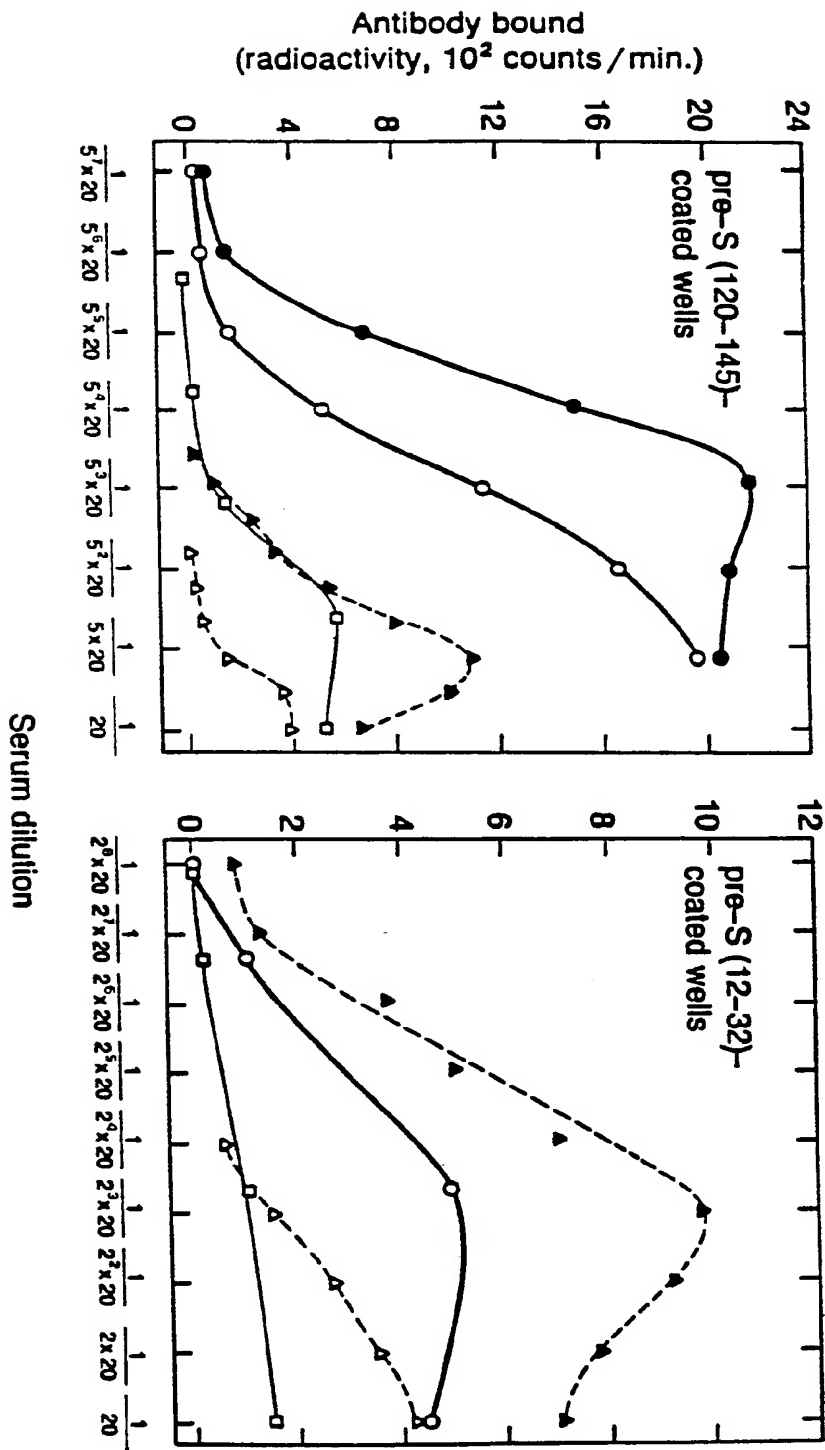


FIG. 11

h107

M/14

0154902

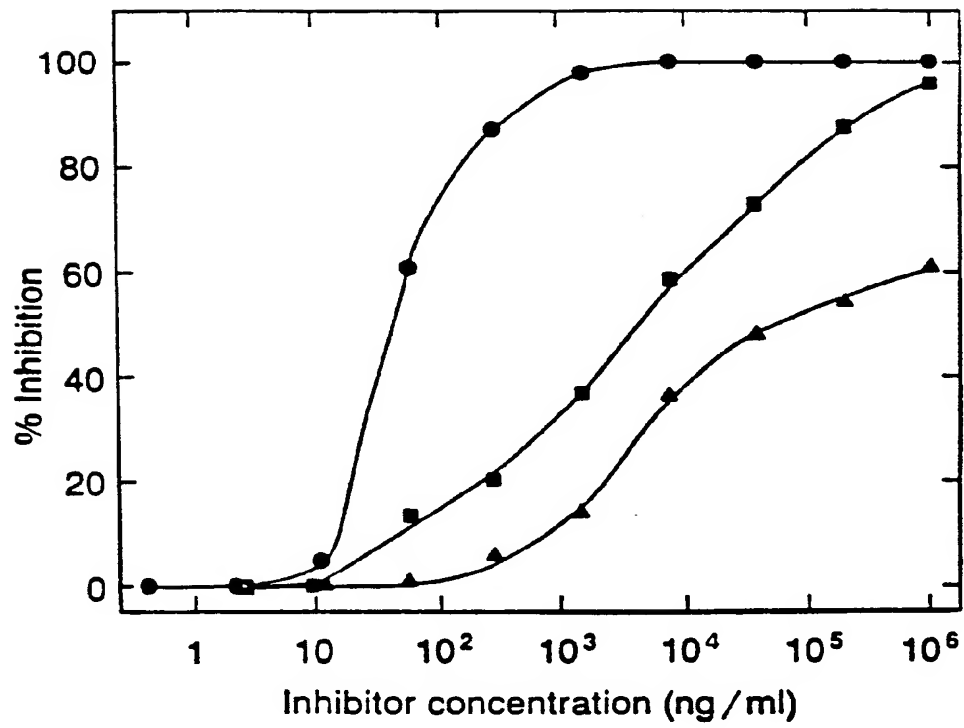


FIG. 12

12/14

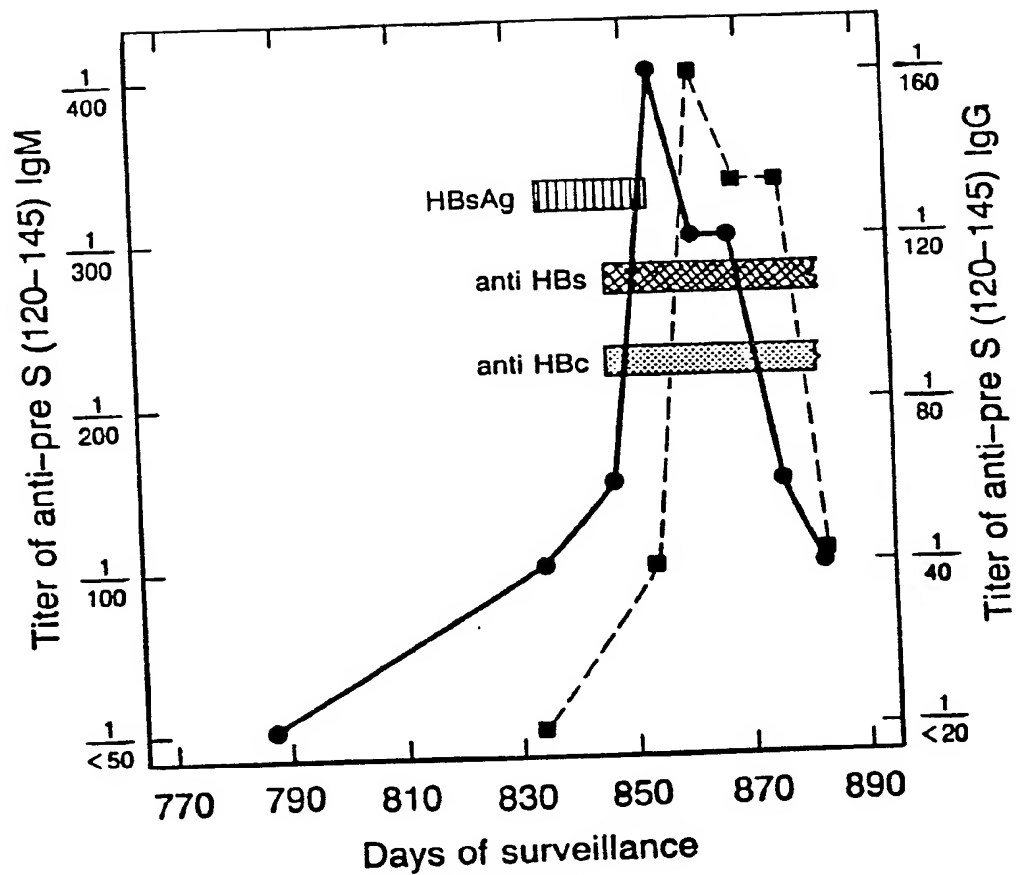


FIG. 13

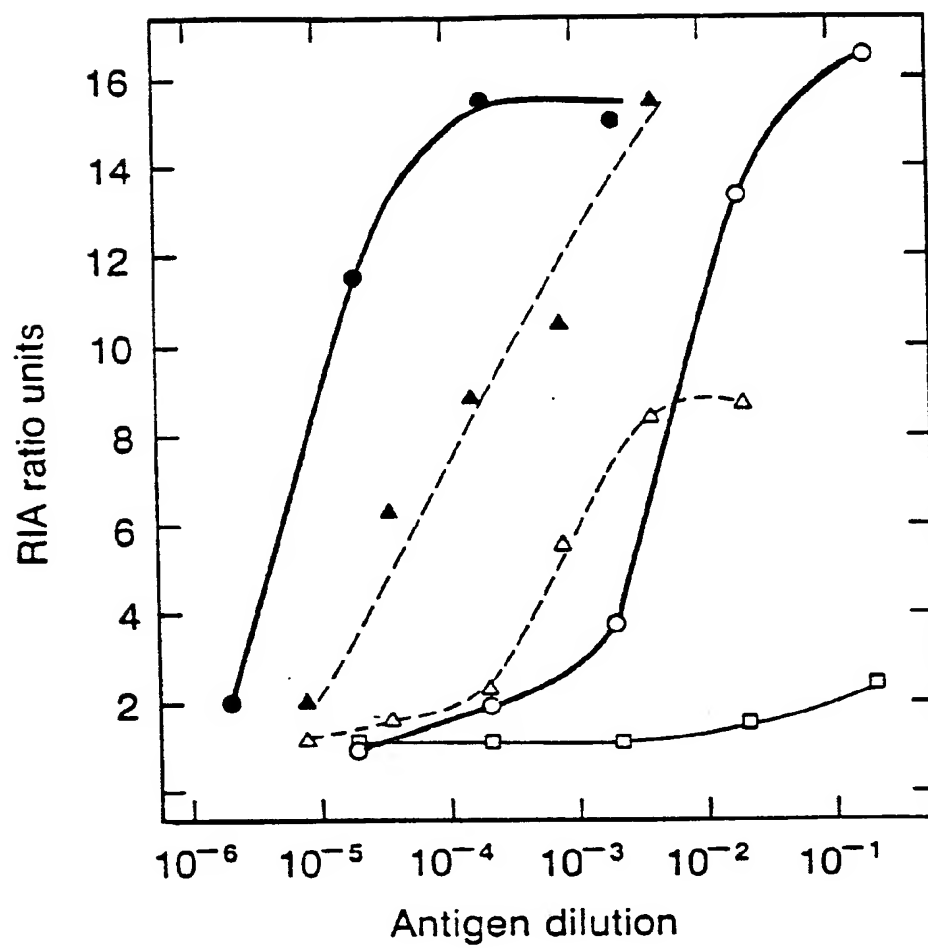
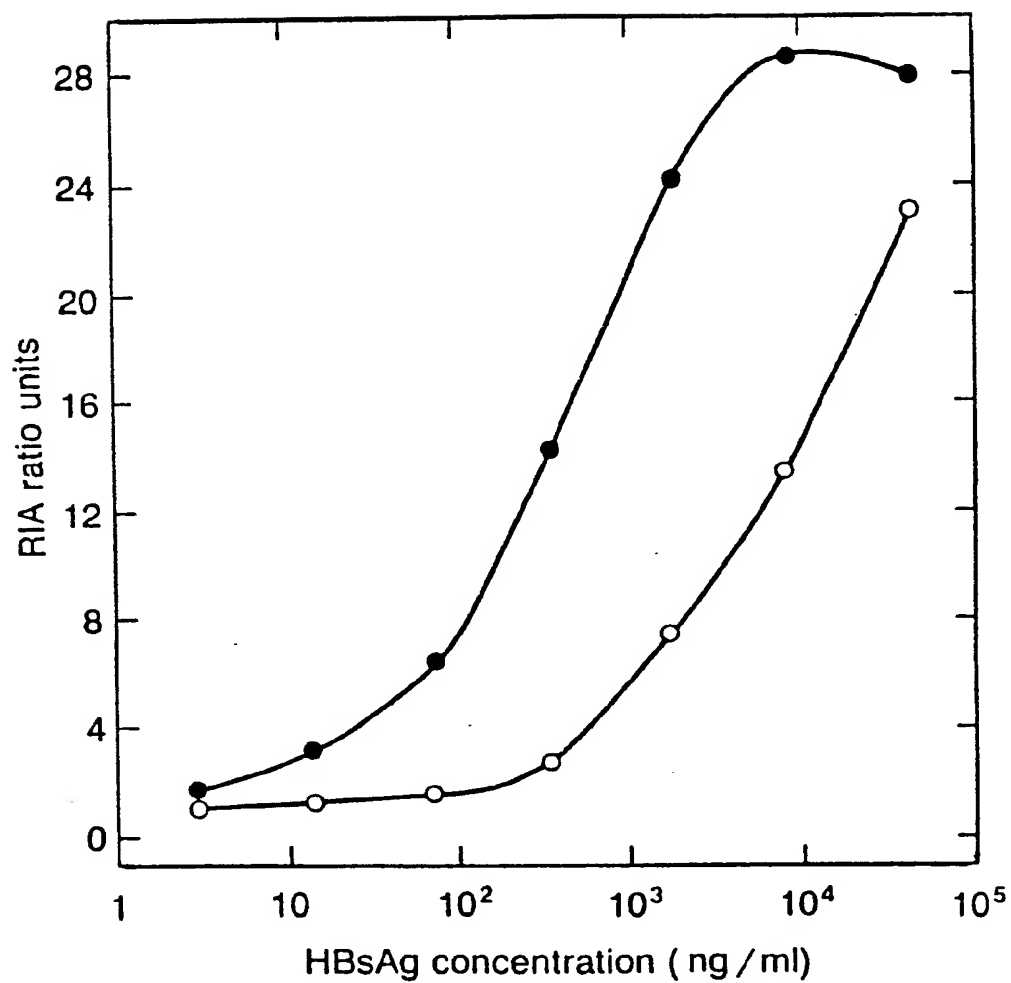


FIG. 14

**FIG. 15**